CANADIAN JOURNAL OF RESEARCH

VOLUME 26 APRIL, 1948 NUMBER 2

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NATIONAL RESEARCH COUNCIL
OTTAWA, CANADA

CANADIAN JOURNAL OF RESEARCH

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For the present, Sections A, C, D, and E are to be issued six times annually, and Sections B and F, twelve times annually, each under separate cover, with separate pagination.

The *Canadian Journal of Research* is published by the National Research Council of Canada under authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research. The *Canadian Journal of Research* is edited by a joint Editorial Board consisting of members of the National Research Council of Canada, the Royal Society of Canada, and the Chemical Institute of Canada.

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Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 26, SEC. C. APRIL, 1948 NUMBER 2

CHROMOSOME BREAKAGE INDUCED BY ABSORBED RADIOACTIVE PHOSPHORUS, P^{32}[1]

BY T. J. ARNASON,[2] ELAINE CUMMING,[3] AND J. W. T. SPINKS[4]

Abstract

Radioactive phosphorus (P^{32}) was made available to individual germinating seeds and seedlings of *Triticum vulgare*, *T. durum*, *T. monococcum*, and *Hordeum distichon*. Each treated plant was provided with either 0.18 or 0.018 microcuries of P^{32}. Both concentrations were effective in causing chromosome breakage and rearrangements. Many aberrant chromosome configurations occurred in microsporocytes of treated tetraploid and hexaploid plants. Only one chromosome aberration was found in *T. monococcum* and none in barley. Structural changes occurred also in chromosomes of vulgare wheat grown in soil to which fertilizer containing P^{32} was added. It is possible that the effectiveness of small amounts of P^{32} in inducing mutations is increased by its inclusion in chromosome molecules. Emitted beta particles would then be shot off very close to the sensitive target. Further, the recoiling atomic nucleus is almost certain to have enough energy to break any chemical bond. In any case since there is a change in atomic number (from 15 to 16) and in valence (P valence 5, S valence 2), molecular bonds must be released in the process.

Introduction

An investigation of the effectiveness of absorbed radioactive elements in inducing mutations in plants was begun in the spring of 1947. A search of the literature yielded no reports on that topic. This was somewhat surprising since a considerable mass of information has been assembled regarding the effectiveness of beta, gamma, and other rays, emitted from an external source, in inducing chromosome breakage and gene mutation (3). By the use of appropriate radioactive elements it is possible to introduce a source of beta or gamma radiation into the living cells themselves and even into the parts known to be of especial importance in heredity: the chromosomes. As will be pointed out in the discussion, some special effects may be expected if the radioactive atoms are in the gene molecules or in the molecules making up the 'backbone' of a chromatid.

The use of absorbed radioisotopes has certain undesirable features, namely: (1) Exact dosage is not very easily controlled and may require rather tedious procedures to determine exactly. (2) It is not possible to stop the dosage at any desired time. (3) Constant vigilance must be exercised to prevent

[1] *Manuscript received December 10, 1947.*
Contribution from the University of Saskatchewan, Saskatoon, Sask.

[2] *Associate Professor of Biology.*
[3] *Research Assistant.*
[4] *Professor of Physical Chemistry.*

[The February issue of Section C (Can. J. Research, C, 26: 1-107. 1948) was issued April 28, 1948.]

contamination of ground, instruments, personnel, etc. (4) The preparation, purification, and standardization of the appropriate solutions involves painstaking and repetitious labor. However, for certain types of work, e.g., treating large numbers of plants just before flowering, it may be more convenient to use solutions of radioactive elements than to use other radiation methods.

Radioactive phosphorus P^{32} was used since it appeared to be particularly well suited for experiments of this kind. Phosphorus salts are readily absorbed by plants and apparently there is free movement of phosphorus within plants (2). This element is a constituent of nucleoproteins, which are abundant in chromosomes and probably in genes (6). Consequently we can expect that some of the disintegrating atoms will actually be those incorporated in the chromosomes, possibly also in the genes.

Materials and Methods

Plant species used as test organisms included the following: *Triticum vulgare* Vill. variety Thatcher ($n=21$), *Triticum durum* Desf. variety Pelissier ($n=14$), *Triticum monococcum* L. ($n=7$), and *Hordeum distichon* L. variety Hannchen ($n=7$).

Seed germination tests (7) had proved that seeds of these species could germinate individually in test tubes containing 0.1 ml. of nutrient solution including as much as 0.18 μc. (microcuries) of P^{32} in the form of disodium hydrogen phosphate. Twenty-four seeds of each species were germinated in the 0.18 μc. solution, another 24 in 0.018 μc. solution, and six in nutrient solution with no active phosphorus. When the solution was absorbed, Knop's nutrient solution with phosphorus omitted was added to each test tube. Measurement of the amount of P^{32} remaining in the test tubes at the end of 13 days indicated that 90 $\pm$ 4% of the active phosphorus had been absorbed by the plants. At this time the seedlings were transferred to 1-gal. crocks containing untreated soil.

When the plants had reached the appropriate stage (about six weeks after seed germination) anthers were collected and fixed with 3 : 1 alcohol–acetic mixture. Microsporocytes were smeared in iron–acetocarmine, the preparations being made permanent by mounting in diaphane.

Pollen mother cells were also obtained from Thatcher wheat plants grown in soil to which had been added 9.6 μc. of P^{32} in the form of disodium hydrogen phosphate for each plant. The cells were then examined to determine whether any chromosome aberrations were present.

Results

A summary of the treatments given and the numbers and chief kinds of chromosome rearrangements observed in microsporocytes is provided in Table I. The following aberrant chromosome configurations were seen: (1) at diakinesis and metaphase I—fragments, chains of three and of four chromosomes, rings of four, and univalents (Figs. 1 and 3); (2) at anaphase

TABLE I

NUMBERS OF MICROSPOROCYTES HAVING VISIBLE CHROMOSOME ABERRATIONS FOLLOWING SEEDLING ABSORPTION OF P^{32}

Species	Number of plants	Microcuries of P^{32} per plant	Number of cells counted	Number of cells aberrant	Number of 'block mutations'	Common rearrangements
Triticum monococcum	6	0	65	0	0	
	24	0.018	170	26	1	Inversion
	24	0.18	112	0	0	
Triticum durum	6	0	54	0	0	
	24	0.018	215	23	3	Intrachromosomal rearrangements
	24	0.18	172	30	4	Inversion; fused sister chromatids
Triticum vulgare	6	0	73	1	0	Chiasma not formed
	24	0.018	160	9	2	Inversion; fragmentation
	24	0.18	230	24	6	Fragmentation; translocation; inversion
Hordeum distichon	6	0	164	0	0	
	24	0.018	72	0	0	
	24	0.18	115	0	0	

and telophase I—split univalents (Figs. 2, 5, and 6) acentric fragments (Figs. 4 and 11), bridges; (3) at anaphase II—bridges (Fig. 9), lagging chromosomes, and fragments; (4) at telophase II and in young microspores—extranuclear chromatin or micronuclei (Figs. 7, 8, 10).

In at least 15 anthers several or many cells had similar or related configurations indicating that chromosome breakage and rearrangement had occurred some cell generations earlier. Such groups of cells are here designated as having a block mutation. These mutations have proved their capacity to survive the mitotic cycle and some of them are likely to be capable of hereditary transmission through spores and gametophytes. It appears reasonable to suppose that chromosome breakage occurred with relatively high frequency in seedlings, when the P^{32} activity was at a maximum and the plant volume relatively small. However, since in some heads particular chromosome rearrangements occurred in only one or two anthers, of several examined, and since single aberrant cells were sometimes found among normal appearing ones it is suggested that breakage and reunion of broken ends continued up to the time when the material was gathered for the cytological investigation. We recognize, however, that small internal chromosome rearrangements might result in noticeable cytological peculiarities in a fraction only of the affected cells.

It is of some interest to note that the two diploid species yielded only one block mutation whereas in the tetraploid durum and in the hexaploid vulgare seven and eight such mutations were found respectively. It may be that

TABLE II

CHROMOSOME ABERRATIONS IN MICROSPOROCYTES OF THATCHER WHEAT SUPPLIED WITH P^{32} IN THE FORM OF Na_2HPO_4 FERTILIZER ADDED TO THE SOIL

Anther number	Meiosis stages	Number of cells counted	Cells visibly aberrant, %	Types of aberrant configurations	Interpretation and conclusion
1	Meta., Ana. I*	64	6.2	Ana. bridge, fragment	Inversion
2	Meta. I	37	2.7	Chain of 3	Translocation and loss of chromosome
3	Diak., Meta. I	32	12.5	Ring of 4	Reciprocal translocation
4	Meta., Ana. I	40	2.5	Bridge and fragment	Inversion
5	Meta., Ana. I	34	0.0	—	—
6	Meta., Ana. I	60	83.3	1–10 univ. (split at anaphase)	Block mutation. Probably intrachromosomal rearrangements
7	Meta., Ana. II	65	0.0	—	—
8	Meta.-Telo. II	75	0.0	—	—
9	Telo.-Tetrads II	41	0.0	—	—
10	Meta. I	60	1.7	Chain of 3	Translocation and loss of one chromosome
11	Meta. I	51	84.3	20 pairs and 1 long univ.	Block mutation. One chromosome lost
12	Ana. II	55	0.0	—	—
13	Ana. I	11	0.0	—	No rearrangement
14	Meta. I	22	9.1	2 unequal univ. 1 univ.	Deficiency
15	Meta. I	18	11.1	Ring of 4 Chain of 4	Reciprocal translocation
16	Diak.-Meta. I	28	92.9	I univ. off plate	Block mutation. One chromosome lost
17	Meta.-Telo. II	64	0.0	—	—
18	Telo. I, Meta. II	19	0.0	—	—
19	Diak. I	10	0.0	—	—

* *I refers to first meiotic division.*

FIGS. 1 TO 11. *Photomicrographs of wheat microsporocytes and spores.* FIGS. 1 TO 6: *T. vulgare.* FIGS. 7 TO 10: *T. durum.* FIG. 11: *T. monococcum.* FIGS. 1 AND 3: *metaphase I in anther number 11, Table II. One univalent at left end. 475×.* FIG. 2: *anaphase I in anther 16 of Table II. Split univalent between chromosome groups. 485×.* FIG. 4: *early telophase I. Split fragment. (0.18 μc. P^{32}). 460×.* FIG. 5: *anaphase I in anther number 6 of Table II. Approximately eight univalents split. 470×.* FIG. 6: *anaphase I. Two split univalents. (0.18 μc. P^{32}). 440×.* FIG. 7: *telophase II. Broken bridge (0.18 μc. P^{32}). 450×.* FIG. 8: *tetrad. Three dense chromatin masses outside nuclei. (0.18 μc. P^{32}). 480×.* FIG. 9: *telophase II. Chromosome bridge. (0.18 μc. P^{32}). 600×.* FIG. 10: *telophase II. Split fragments. (0.018 μc. P^{32}). 425×.* FIG. 11: *anaphase I. Lagging acentric fragments. (0.018 μc. P^{32}). 460×.*

PLATE I

the greater total chromosome length in the polyploid nuclei has simply provided more chances for breakage to occur or it may be that many kinds of rearrangement are cell lethal in diploids but not in polyploids, e.g., a homozygous deficiency may be fatal to diploid but not to polyploid cells since, in the latter, genes similar to those lost are present in other chromosomes.

Of the 19 anthers examined from the Thatcher wheat plants grown in soil to which P^{32} had been added, six had second division figures and tetrads only. Several types of rearrangements might escape detection at these stages. Block mutations were found in three of the remaining anthers. In two of these (Figs. 1, 2, 3) one chromosome had been lost; in the third (Fig. 5) there was a large but varying number of unpaired chromosomes (up to 10). Possible causes of this failure to pair include extensive intrachromosomal rearrangements and mutation or loss of a gene influencing pairing. A summary of the cytological results is provided in Table II.

The cytological collections were carried on for three days. On the day following the last collection the plants were harvested, dried, weighed, and ashed; counts were then made of the amount of active P^{32} present. Roots were not included. A complete set of anthers was collected from one plant. The mass of these anthers (air-dried) was determined to be 0.0035 gm. The average mass (dry weight) of a plant as harvested was 1.25 gm. The total amount of activity in an average plant was 0.04 μc. In the tested anther set the total amount of active P^{32} was 0.0023 μc. Based on these figures the mass of the anthers is estimated at 0.28% of the mass of the whole plant; the P^{32} present in the anthers is a much larger part of the whole, being 5.7% of the total activity in the plant.

Discussion

The preliminary results reported in this paper and previously mentioned in a brief note in Science (1) indicate that small doses of P^{32} absorbed by young plants and present in meristematic cells are effective in causing numerous chromosome breaks. Only a small fraction of the breaks induced in apical meristems of the plants are likely to be represented and identifiable in microsporocytes. Breakage having cell lethal effects and breakages in cells not in the direct cell lineage of anthers will not be found. Small changes are likely to be overlooked. The effectiveness of small amounts of absorbed P^{32} may be ascribed in part to the fact that phosphorus is a constituent of many proteins and in particular of nucleoproteins, which are abundant in chromosomes (6) and probably in genes. Beta particles that are emitted by atoms located in the gene molecule or elsewhere in chromosomes must have, because of the closeness of the firing point to the target, a higher probability of passing through gene molecules or the protein backbone (5) threads of chromatids than beta particles emitted from a more distant source. Furthermore, the recoiling atom nucleus is almost certain to have sufficient energy to break any chemical bond (4). According to Lea (3) 4 ev. of energy is sufficient to dissociate the C—H bond in a molecule. A change in atomic number

occurs when P^{32} emits an electron, $_{15}P^{32}$ becoming $_{16}S^{32}$ with accompanying change in valence from 5 to 2. It is apparent that in any case the new atom cannot maintain all the bonds joining to the original P atom. Some of the molecular bonds involving P^{32} must break when the radioactive transformation occurs. The occurrence of these phenomena may make radioactive substances such as P^{32}, which may be incorporated in gene or chromosome molecules, highly effective in producing chromosome breaks and possibly potent in inducing gene mutations.

Acknowledgments

It is a pleasure to acknowledge assistance from the following: the National Research Council of Canada, Dean B. Cowie of the Carnegie Institution of Washington, and Mr. N. Ferrier of the Biology Department.

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TETRAPLOID *TARAXACUM KOK-SAGHYZ*

IV. COMPARISON OF SECOND GENERATION FAMILIES[1]

By M. W. Bannan[2]

Abstract

The general similarity in root weight between the populations from reciprocal crosses of tetraploids, the differences between unrelated families, and the divergence between the progeny of large-rooted and small-rooted parents together indicate the operation of genetic factors in root development. Number, size, and shape of leaves likewise appear to be strongly influenced by heredity. As a rule, vigorous rosettes, large roots, and a sparsity or absence of first-year flowering are associated, but diverse assortments of these characteristics occur in some families. Since sparse flowering favors root enlargement, the later blooming and less floriferous tetraploids have an advantage over diploids in that respect. On the whole, random tetraploids possess wider but markedly fewer leaves, slightly smaller leaf area, bloom later and much more sparsely during the first year, and produce larger and more robust roots than diploids.

Introduction

Although kok-saghyz is poorly adapted for genetical studies because of its incompatibility mechanism, the potentialities of the species as a rubber producer have rendered important the acquiring of information on the heritability of such characteristics as root size and rubber content. While diploids are more suitable for such studies than tetraploids, extensive experimentation with both has not been possible, and since the writer's investigations have been concerned mainly with tetraploids, these were used for crossing. First generation tetraploids derived from colchicine-treated parents were selected for root size, segregated into two groups, and the largest and smallest crossed among themselves to yield a second filial generation. The various morphological characters of the families in this second generation are described and comparison made both with each other and, in the matter of root size, with the parents.

Methods

After removal from pots in the autumn of 1945 the roots of first generation tetraploids were weighed, the largest and smallest repotted and sunk in the plot for overwintering. Early the following spring the plants were removed to the greenhouse and the largest and smallest crossed among themselves. As flowering waned the roots were once again weighed and the rubber content determined. From the seed lots so obtained selections were made on the basis of parental root size and rubber content, some of the best and poorest combinations being chosen. The achenes, which were stored during the winter of 1946–47 at room temperature, were sorted in April 1947, the empty hulls removed, and the apparently sound specimens weighed.

[1] *Manuscript received November 26, 1947.*

Contribution from the Department of Botany, University of Toronto, Toronto, Ont. with assistance from a grant in aid of research furnished by the University of Toronto.

[2] *Assistant Professor in Botany.*

These were placed on moist filter paper in Petri dishes without pregermination chilling. When the radicles protruded, the seedlings were set out singly in sterile soil in 2½ in. pots. In late May and early June the seedlings were transplanted in the garden plot at 7 in. spacings in rows alternately 12 and 18 in. apart, the maximum spacing in the limited area available. As the plants grew, records were kept of the number of leaves, diameter of the rosette, and head production for all plants, and estimates made of the seasonal march of leaf area for plants representative of the different families. In early October the roots were carefully removed from the soil, washed, and weighed, the weights including the defoliated crowns.

Control diploids were grown from mixed achenes harvested in 1946. One-half the plants were allowed to flower and produce achenes, and the remainder were disbudded. In the hope of determining the effect of age of achenes on plant development, parallel series of tetraploids and diploids were grown from mixed 1945 achenes, and two lots of diploids from 1945 and 1944 achenes. Unfortunately, for reasons beyond control, these lots were of different origin. The 1944 achenes were collected from open-pollinated plants grown locally; the 1945 achenes were provided by Dr. L. Truscott of the Ontario Agricultural College; and those of 1946 were gathered from open-pollinated plants originating from the 1945 achenes. Possibly because of genetic variability associated with difference of origin, these series failed to provide information of comparative value.

Rosette Characters

Data on the number of leaves and diameter of the rosette at maximum size are given in Table I for various $4n$ and $2n$ series. In the tetraploids the biggest rosettes were usually found among the descendants of large-rooted parents, though considerable interfamily diversity occurred in this group, 1690 × 2076 possessing rosettes more than twice as leafy as 2073 × 1845. Families of small-rooted lineage, such as 2062 × 2057, tended to have less vigorous rosettes, the diameter especially being noticeably reduced. On the whole, however, the differences in rosette size between derivatives of large or small plants were not as great as the differences in root size. Perhaps the most striking feature of the data in Table I is the general similarity in rosette size between the reciprocal crosses. For instance, in the case of 2073 × 2050 and 2050 × 2073 (two crosses) the average number of leaves at the maximum was 79.1, 79.6, and 80.9 and the average diameter 13.9, 14.1, and 14.7 in. The similarities between the reciprocal crosses, which stand in contrast to the interfamily and intergroup diversity, may be interpreted as indicative of genetic influence on rosette development.

The seasonal march of leaf area is illustrated in Fig. 1 for approximately 90 plants representing nine crosses. Leaf area followed a generally parallel course for each cross and its reciprocal, but differed considerably in the various families. In this connection it should be explained that the early separation of the families into two groups, as shown in the figure, was due to slight

TABLE I

DATA ON FIRST-YEAR, TETRAPLOID FAMILIES AND DIPLOIDS FROM MIXED ACHENES, GROWN IN THE GARDEN PLOT (1947)

Description	Av. root weight parents, gm.	Av. weight achenes, mgm.	Germination, %	No. of plants	Av. no. of leaves	Predominant leaf type	Av. diam. rosette, in.	Av. no. of heads	Fresh root weight, gm.
4*n* 2073 × 2061	64.7	0.92	67	8	66.0	IV–V	17.8	24.8	57.1
2061 × 2073	64.7	0.56	66	32	56.0	IV–V	15.5	10.9	46.5
2073 × 1845*a*	64.5	0.77	44	14	49.4	IV	13.9	1.5	40.1
2073 × 1845*b*	64.5	0.80	67	26	48.6	IV–V	13.1	3.1	49.1
1845 × 2073	64.5	0.68	18	6	51.0	IV	12.6	7.0	32.0
2056 × 2073*a*	63.0	0.63	79	25	74.0	IV–V	13.9	12.9	49.7
2056 × 2073*b*	63.0	0.41	81	30	67.5	IV	13.2	12.0	43.1
2073 × 2050	57.5	0.89	97	27	79.1	IV	13.9	4.1	50.8
2050 × 2073*a*	57.5	0.44	77	39	79.6	IV	14.1	5.3	48.2
2050 × 2073*b*	57.5	0.49	79	36	80.9	IV	14.7	11.8	47.8
2056 × 2076	43.0	0.50	92	26	79.5	III–IV	16.8	11.2	30.3
2050 × 1845	42.2	0.54	35	11	59.0	III–IV	13.8	3.2	35.6
1845 × 2050	42.2	0.81	57	26	77.0	III–IV	14.2	16.0	41.7
2056 × 2050	41.5	0.54	90	46	80.6	IV	12.8	8.8	37.7
2061 × 1690	40.5	0.62	71	25	67.2	IV	13.8	5.1	45.1
2050 × 2076	37.7	0.61	55	21	76.6	III	16.5	12.1	34.2
2076 × 2050	37.7	0.81	93	23	78.4	III	16.8	27.5	28.0
2076 × 1690	35.2	0.86	94	23	86.0	IV	18.4	11.3	41.7
1690 × 2076	35.2	0.59	81	12	123.8	IV	16.7	1.5	49.3
4*n* 1780 × 2057	9.5	0.52	60	19	78.2	III–IV	15.7	23.5	31.7
2062 × 2057	9.0	0.66	41	9	51.9	III–IV	8.5	0	18.3
2069 × 1614	8.7	0.70	85	29	51.0	III–IV	12.1	4.4	26.1
1641 × 2062	8.7	0.76	41	17	73.5	III–IV	12.5	6.8	34.8
2062 × 1641	8.7	0.63	56	9	61.9	IV	11.1	0	26.7
4*n* mixed 2-yr. achenes	—	0.72	88	39	63.0	—	12.8	17.9	35.9
2*n* mixed 1-yr. achenes (disbudded)	—	0.48	94	35	92.3	—	11.2	87.1	15.0
2*n* mixed 1-yr. achenes (flowering)	—	0.48	94	39	71.6	—	10.8	45.1	12.7
2*n* mixed 2-yr. achenes	—	0.41	66	57	116.6	—	12.6	88.4	24.8
2*n* mixed 3-yr. achenes	—	0.41	35	17	133.0	—	11.6	25.6	37.7

differences in greenhouse illumination and time of transplantation outside. After transplanting, all plants grew rapidly and, in the case of the families investigated, leaf area expanded at more or less similar rates. The feature of particular significance when contrasting the families is the divergence after the attainment of maximum size. It will be observed that the reciprocals 2050 × 2076 and 2076 × 2050 conformed to the usual pattern for kok-saghyz, leaf area falling off sharply during the rest period in late summer and early autumn. In contrast the rosettes of 2073 × 2050 and reciprocal underwent continuous replenishment so that leaf area remained steady until harvesting. Scarth *et al.* (17), in their studies on summer dormancy in diploid kok-saghyz, came to the conclusion that the growth cycles in kok-saghyz were due to an internal rhythm. They believed that a setback in rosette size took place at a definite stage in ontogeny and this recession could be modified but not pre-

vented by environmental manipulation. The observations on tetraploids recorded in the present paper indicate the possibility of family differences in the rhythm of growth that would appear to have a genetic basis.

Among the random diploids, those grown from three-year-old achenes had the leafiest rosettes and the derivatives of one-year-old achenes the smallest

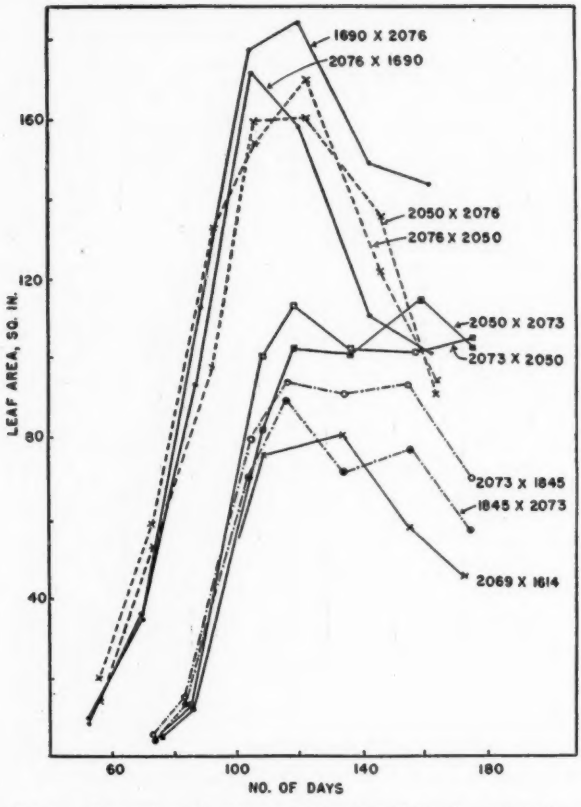

FIG. 1. *Seasonal march of leaf area in representative 4n reciprocal crosses.*

(Table I). In the latter, disbudded plants produced leafier and wider rosettes than those allowed to flower, but the size in both disbudded and flowering plants was unaccountably small. In the parallel series of tetraploids and diploids grown from mixed two-year-old achenes, the latter had 72% more leaves than the former. This profusion of leaves in the diploids more than compensated for the small size of individual leaves, so that at maximum development leaf area slightly exceeded that of comparable tetraploids (Fig. 2). A similar slight superiority among diploids was reported in a previous

paper (3) for potted plants. The late-summer recession, however, was less drastic in the tetraploids.

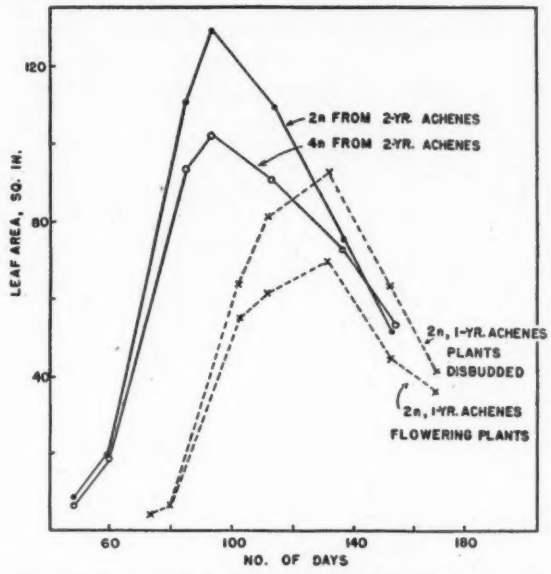

FIG. 2. *Seasonal alterations in leaf area in diploids and tetraploids grown from mixed two-year-old achenes, and disbudded and flowering diploids from one-year-old achenes.*

Root Size

As shown in Table I the progeny of large-rooted tetraploids developed robust roots, although there was in most cases some regression from the parental size. Conversely the descendants of small-rooted plants produced roots below average size, although superior to the parents. The average root weights in the two groups were quite distinct, being 42.0 ± 7.7 gm. for the progeny of large plants and 26.7 ± 6.3 gm. for the offspring of small plants. The root weights differed in various lots, but it is noteworthy that they were generally similar in the cross and its reciprocal. For instance, in 2050 × 2073 (two crosses) and the reciprocal 2073 × 2050 the average weights were 47.8, 48.2, and 50.8 gm. The greatest difference occurred in 1845 × 2073, where the average weight was only 32 gm. as contrasted to 40.1 and 49.1 gm. for the reciprocals. Here, however, special circumstances existed. The achenes of 1845 × 2073 germinated more poorly than in any other lot, and few seedlings developed. As these neared maturity their numbers were further reduced by rot, the percentage loss being greater than in other groups and much exceeding the 1% loss for the plot as a whole. As a consequence of these unfortunate developments only six plants were harvested, a number much too small for

the data to have significance. On the whole, the results from the various crosses point to hereditary influence on root size.

The root habit of the tetraploids was highly variable (Fig. 3). Some possessed stout tap roots with only feeble secondaries. Others had coarse

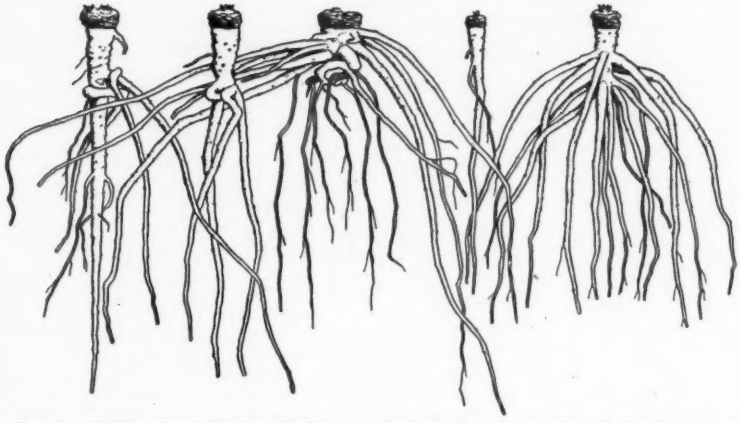

FIG. 3. *Types of root systems in first-year tetraploids. From left to right weights were 64.5, 43.0, 81.0, 15.5, and 57.0 gm.*

fibrous systems with numerous secondaries equalling or surpassing the tap root. Occasionally a tap root was not discernible. Analysis of the data on configuration of the root systems failed to reveal outstanding interfamily differences, except that the crosses with the weightier roots generally had better developed secondaries.

Among the diploids the largest roots came from three-year-old achenes, the smallest from one-year-old achenes. Disbudding produced a slight increase in root size, but even the disbudded plants developed abnormally small roots. For some reason the derivatives of 1946 achenes were subnormal in all recorded growth characteristics such as leaf area, head production, and root size. They were inferior to random diploid plantings in previous years, to the parental series, and to the current year's plants grown from the same seed lot as their parents. The relative failure of this series could not be attributed to environmental factors in the garden plot. Rather it would appear to be due to a preponderance of genetically inferior individuals, despite the fact that some 74 plants were involved and the achenes were produced by open pollination of an equal number of parental plants, themselves grown from mixed achenes. Whatever the explanation may be, it is clear that the data from these plants have no comparative value. We would seem to have here an illustration of the fallacy of deriving conclusions from a small number of plants, especially when the species is as variable as kok-saghyz.

For comparison of 4*n* and 2*n* root weights in mixed populations reference may be made to Table IV. Here it will be seen that the average root weight of tetraploids grown in 1947 from two-year-old achenes was 35.9 gm., and that of diploids, 24.8 gm. These values compare with 31.3 gm. and 26.9 gm. for plantings of the previous year from similar seed lots, then one year old. It should be noted that the 1946 plantings were in 8-in. pots, those of 1947 in the garden plot, an environment more favorable to growth.

Correlation of Morphological Characters

General relationships between rosette size, flowering, and root weight such as reported previously (3) were noted again in the current plantings. The data are presented in Table II for two groupings of 4*n* crosses with slightly different combinations of growth characteristics, and for diploids grown from 1945 achenes. In each case there was positive correlation between number of leaves at maximum development, diameter of the rosette, and root weight, and an inverse relationship between first-year flowering and plant size.

TABLE II

RELATIONSHIPS BETWEEN FRESH ROOT WEIGHT, HEAD PRODUCTION, NUMBER OF LEAVES, AND SIZE OF THE ROSETTE IN FIRST-YEAR DIPLOIDS AND TETRAPLOIDS

Description	Root weight, gm.	No. of plants	Av. no. of heads	Av. no. of leaves	Av. diam. of rosette, in.
4*n* (var. crosses)	To 19.9	14	19.4	46.5	11.9
	20–39.9	59	18.0	64.0	12.7
	40–59.9	86	7.0	77.3	14.2
	Over 60	50	0.7	98.3	15.8
4*n* (var. crosses)	To 19.9	25	25.0	61.5	12.5
	20–39.9	75	16.4	76.2	14.8
	40–59.9	43	8.2	86.0	15.8
	Over 60	8	0	99.1	16.1
2*n* (mixed 2-yr. achenes)	To 9.9	6	93.0	88.3	11.1
	10–19.9	20	93.9	89.5	11.5
	20–29.9	19	107.7	116.3	12.8
	Over 30	12	40.4	164.8	13.0

TABLE III

FRESH ROOT WEIGHT AND LEAF TYPE IN FIRST-YEAR PLANTS

Series	No. of plants	Av. root weight in grams of plants with leaf types as indicated			
		I and II	III	IV	V
4*n* (var. crosses)	272	24.0	30.3	37.4	52.1
4*n* (var. crosses)	261	24.5	30.8	46.3	53.6

TABLE IV

COMPARISON OF 1947 AND 1946 PLANTINGS FROM A MIXED SEED LOT

Description	Site	No. of plants	Av. no. of leaves	Av. diam. of rosette, in.	Av. no. of heads	Av. root weight, gm.
4*n* 1947, 2-yr. achenes	Field	39	63.0	12.8	17.9	35.9
1946, 1-yr. achenes	Pots	79	37.0	11.7	4.0	31.3
2*n* 1947, 2-yr. achenes	Field	57	116.6	12.6	88.4	24.8
1946, 1-yr. achenes	Pots	58	97.8	12.3	37.1	26.9

TABLE V

FIRST-YEAR FLOWERING IN TETRAPLOIDS AND DIPLOIDS

Type	Age of achenes	No. of plants	Plants flowering, %	Av. head production per plant
4*n*	2 years	39	48.5	17.9
2*n*	2 years	57	95	88.4
4*n*	1 year	565	28.5	9.3
2*n*	1 year	77	90	66.1

While the general relationships were as described above, individual plants varied considerably, some possessing diverse combinations of the different characters. This deviation was sometimes general for a whole family. For instance, in the reciprocals 2050 × 2076 and 2076 × 2050, leaf area was large (Fig. 1), considerably above normal for the various 4*n* populations, but root weight was definitely below average for the group (Table I). In contrast, the leaf area in 2073 × 2050 and reciprocal was much smaller (Fig. 1), but the roots were bigger. Within each of these families, however, the majority of the plants conformed to the general pattern, the largest tending to have the biggest roots. It would appear from the data that rosette size and root weight are both heritable, and that while they are usually associated, some individuals and families possess different assortments of these characters.

The relationships between root weight and the seasonal behavior of the rosette were varied. In most cases the rosettes of large-rooted plants expanded faster and reached maximum size later than those of small-rooted sibs. In Fig. 4 it will be noted that the rosettes of the large plants in 2050 × 2073 and reciprocal, with roots exceeding 60 gm., continued to expand until harvesting, whereas the small plants, with roots under 40 gm., went into a late-summer recession. In 1690 × 2076, on the other hand, the rosettes of both large and small plants underwent reduction in late summer, but the onset of the rest period was delayed and the recession was of lesser extent in the large plants. Yet another condition was observed in 2050 × 2076

and reciprocal. Here the large-rooted plants, as usual, had much greater leaf area than the small-rooted sibs, but maximum size was attained more or less simultaneously and subsequent reduction was of similar degree in both cases. Although much variation occurred, both in individuals and in families, there was a general tendency for the rosettes of the large-rooted plants to grow faster, attain maximum size later, and to recede less in the late summer, as illustrated by var. crosses, Fig. 4.

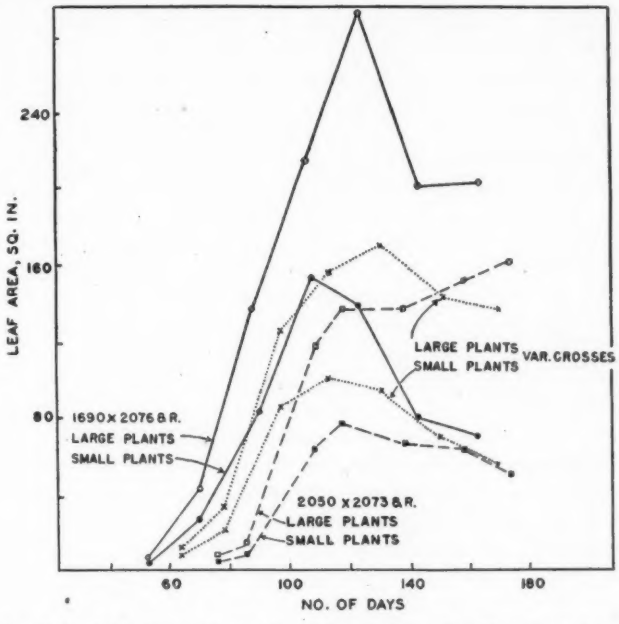

FIG. 4. *Estimated leaf area of large plants with roots exceeding 60 gm. and of small plants possessing roots under 40 gm. in 4n reciprocal crosses. Total, 167 plants.*

Leaf shape and nature of margin were highly variable, but tended to cluster about modes specific for each 4*n* family. As noted in a previous article (3), where the literature is reviewed, the observations of various authors indicate that leaf shape is determined by the interplay of ontogenetic, environmental, and genetic factors. As a rule 4*n* progeny at maturity bore leaves resembling the parental types, but within each family some fluctuation occurred, with the largest plants tending to have the more deeply lobed leaves. Data on this relationship are given in Table III. Here the leaves are divided into five categories, with the lobing increasing from Types I to V as illustrated previously (3). The various 4*n* crosses are divided into two groups, the first with leaves of Types III–IV predominating, the second with Types IV–V prevailing. In both groups the plants with the more deeply lobed leaves

(Types IV and V) tended to have larger roots. At the same time, of course, much individual variation occurred.

As referred to earlier, flowering has an inhibiting effect on root development. Scarth *et al.* (17) observed that disbudded diploids surpassed flowering plants, and Roberts and Struckmeyer (16) found flowering plants in a number of genera had smaller roots in relation to tops than nonflowering plants. Russian investigators have published differing reports on 2*n* kok-saghyz (see Krotkov (9)). In both tetraploids and diploids described in the present paper plants with the biggest roots tended to flower least (Table II). Results from the 1947 plantings thus agree with those of 1946 (3) in demonstrating an inverse relationship between flowering and root development. The cumulative evidence indicates that two complexes of factors are involved in this relationship. First, the internal circumstances (hormone balance, etc.) that bring about flowering appear to have a deleterious effect on vegetative growth. Second, structural and food materials utilized in flower and seed production are not available for continued root development. That the first of these factors might be the more important is suggested by the results from the diploids grown from the year-old achenes. Here root weights were 12.7 gm. for flowering plants, 15.0 gm. for disbudded plants, and 25.0 gm. for nonflowering plants. However, it should be emphasized that the series of 74 plants was too small for the results to be conclusive. Evidence is required from other plantings before it is possible to assess the relative potency of the two factors.

It was hoped that the diploid plantings from one-, two-, and three-year-old achenes would provide information on the influence of age of achenes on plant development. Unfortunately, the obviously subnormal development of both vegetative and reproductive organs in the plants from year-old achenes prevented such comparison. In the 1946 plantings (3), it was noted that aging of the achenes for a second year induced a sharp reduction in flowering. This effect was not apparent in the 1947 plantings, both diploids and tetraploids from two-year-old achenes blooming well. The fact that the 1947 plants were transplanted in the garden plot, whereas those of 1946 were confined to 8-in. pots, although sunk outside, may have been a factor in maintaining a high level of flowering in the 1947 derivatives of two-year-old achenes. Cumulative evidence from various plantings during the past few years indicates that field conditions favor both more vigorous vegetative growth and greater flowering. Some data bearing on this question are presented in Table IV. Here it will be noted that both tetraploids and diploids produced bigger rosettes and more heads when removed from pots, but the response of the tetraploids to the more favorable environment surpassed that of the diploids. Root weight of the tetraploids showed a slight increase, that of the diploids a small decrease, possibly because of the upsurge in head production. Whatever the factors may be, the 1947 series of plot-grown plants did not exhibit the sharp recession in flowering among the derivatives of two-year-old achenes such as noted in the potted plants of 1946. However,

in the 1947 plants grown from three-year-old achenes, a decided falling off in flowering did take place (Table I). Germination in this group was reduced to one-third by the three years' aging of the achenes, but vegetative development of the survivors was not impaired. Actually the rosettes were leafier and the roots larger than in the other series, doubtless due in part to the reduced flowering. For the acquiring of satisfactory information on the effect of age of seed on plant growth it would seem necessary to repeat a number of specific crosses in successive years, in order that seed lots having a more or less similar range of genetic variability would be available for simultaneous planting.

The influence of seed size on plant growth has been investigated in many genera. The literature, reviewed by Oexemann (15) and Spurr (20) indicates that increased seed weight generally results in more vigorous initial growth but does not have a profound influence on size of plants at maturity. The writer came to a similar conclusion in the case of kok-saghyz (3). In the $4n$ plantings described in the present article achene weight sometimes differed considerably between the cross and its reciprocal (Table I) so that there was here perhaps a better basis of comparison than when the achenes were selected from a mixed seed lot. In four families the bigger achenes produced larger plants, in two the reverse was true, and in the remaining two cases the results were inconclusive. On the whole it would appear that if achene weight did have a bearing on ultimate plant size, its effect was of minor importance as compared with that of other factors.

Discussion

The general similarity in average root weight between $4n$ reciprocal crosses, the differences between unrelated families, and the divergence between the progeny of large-rooted or small-rooted parents together indicate the existence of genetic factors in root development. Rosette size likewise appeared to be influenced by heredity. As a rule these characteristics were associated, plants with vigorous rosettes generally producing roots of corresponding size, but in some individuals and occasionally in whole families, different assortments of these characters occurred. Even in such cases, however, trends within the family were of the usual type.

The relationship between flowering and root development was of an inverse nature, the largest tetraploids generally bearing few or no inflorescences during the first year. Mashtakov (10), working with crowded diploid kok-saghyz, reported that the flowering plants produced the biggest roots, but other Russian authors disagreed (see Krotkov (9)). Scarth *et al.* (17) observed that disbudded $2n$ plants surpassed those allowed to produce seed, and Roberts and Struckmeyer (16) found nonflowering plants in a number of genera to have consistently larger roots than flowering plants. Among our diploids there was a general tendency for the large-rooted plants to be less floriferous, and in the tetraploids the trend was more definite. The bulk of the evidence indicates that extensive flowering has an inhibiting effect on

root enlargement, or in other words the internal conditions associated with the reproductive phase retard vegetative growth.

An outstanding feature of tetraploids in the Toronto plantings has been their relatively sparse first-year flowering. In this regard our observations are at variance with those of Navashin *et al.* (13) who reported no difference between $4n$ and $2n$ kok-saghyz in proportion of plants flowering. Numerous authors (4, 6, 11, 12, 14, 18, 19, 21) have remarked on the slower maturing and later inception of blooming of tetraploids. Similar observations were recorded in the local plantings of kok-saghyz. The average interval between soaking of the seed and the first opening of capitula was 68 days in diploids grown from mixed two-year-old achenes and 82 days in the parallel series of tetraploids. As regards degree of floriferousness, the proportion of flowering plants and the average head production per plant were both decidedly lower in the tetraploids (Table V). It should be emphasized that this has been true for all local $4n$ plantings, whether in pots or in the field (2 and 3).

The reduction in first-year flowering among tetraploids is highly significant because of its bearing on root growth. Since profuse flowering and seed production adversely affect root enlargement the tetraploids have an advantage over diploids in their sparse flowering. The biochemical basis of the relationships between vegetative and reproductive phases of growth has been the subject of considerable investigation. Some evidence has been adduced in support of the hypothesis that a specific floral hormone, which Čajlachjan (5) termed "florigen", is responsible for the onset of flowering, though attempts to extract it have met with failure and some investigators doubt its existence as a specific hormone. Galston (7) has raised the question of the possibility of a functional association of various growth regulating hormones in plants. He suggested that in such an association "florigen", assumed to promote flowering, was antagonistic to auxin favoring vegetative growth. Whatever the nature of the various substances having to do with growth may be, it would appear that the balance between these substances was more favorable to root development and less conducive to flowering in tetraploids than in diploids. As well as being affected by chromosome number it would also seem that this balance was influenced by heredity and a succession of environmental factors that probably begin with such circumstances as age of the seed and its pregermination treatment. As regards auxin, Gustafson (8), Smith (19), and Avery and Pottorf (1) have reported lower concentration in tetraploids than in diploids, but more analytical work is required before general conclusions may be drawn. Obviously, such analysis should be accompanied by careful observations on morphology.

Acknowledgments

This paper is the fourth report on a project assisted initially by the National Research Council, and latterly by a grant in aid of research provided by the University of Toronto. For assistance thanks are due to Miss B. E. Whalley.

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STUDIES OF CANADIAN THELEPHORACEAE

I. SOME NEW SPECIES OF *PENIOPHORA*[1]

By H. S. Jackson[2]

Abstract

In connection with a general taxonomic study of the resupinate Thelephoraceae of Canada, a number of forms have been encountered that appear to be undescribed. In this contribution nine such forms falling in the genus *Peniophora* are described and illustrated as probable new species.

Introduction

During the past 15 years the writer has had occasion to examine well over 7000 collections of resupinate Thelephoraceae from all parts of North America. Among such a large number of specimens it is perhaps to be expected that forms have been encountered that appear to be undescribed. Such forms have been accumulating for several years but available time for this study is necessarily limited and most of that time has so far been spent in attempting to become familiar with the old species, both European and American. It is only recently that the writer has developed sufficient confidence to venture to describe as new any of the odd forms that have been encountered.

Our knowledge of the Thelephoraceae of North America, particularly the resupinate forms, is still in a pioneer stage. Much remains to be done in straightening out the confusion that has resulted because of inadequate knowledge of the European species on the part of American mycologists. The writer is fully aware that to propose new species in this group involves considerable risk of merely adding to an already overburdened synonomy since there are many described species in the literature concerning which little is yet known. It has become evident, however, that it is impracticable to hope for complete knowledge of all the forms that have been recorded, many of which have been inadequately described. It is felt that progress will be better served by furnishing accurate illustrations and carefully prepared descriptions of forms that appear to be distinct after thorough study and the elimination of all the better known described species. Included in the present contribution are descriptions and illustrations of nine species of *Peniophora*, all of

[1] *Manuscript received December 18, 1947.*

Contribution from the Department of Botany, University of Toronto, Toronto, Ont. This study was carried out with the assistance of a grant in aid of research furnished by the University of Toronto.

[2] *Professor of Mycology and Head, Department of Botany.*

The writer is indebted to Miss Margaret H. Thomson for the preparation of all but one of the Latin diagnoses and to Miss Charlotte M. Dill for preparing the drawings. Miss E. Ruth Dearden has assisted in the final preparation of the descriptions and Dr. D. P. Rogers has furnished the description for P. cymosa and has read the manuscript.

which have been found in Ontario, which to the best of our knowledge appear to be undescribed.

The genus *Peniophora* is here interpreted in the generally accepted sense as including those species of corticioid resupinates having spores with essentially colorless, usually smooth walls, and that have cystidia in the hymenium. From a general survey of the species included in the genus, it is evident that to place the main emphasis on the character of presence of cystidia results in bringing together several groups of species that show little relationship between groups. In some species or groups it is evident that the real relationship is with species now included in *Corticium*, also a large collective genus, which as currently interpreted, includes most of the simpler resupinates of the group having no special inclusions in the hymenium other than gloeocystidia. In the case of other species it is evident that their relationship is with forms now included in one or another genus of resupinate Hydnaceae. The whole group of resupinate forms among Homobasidiomycetes is obviously in need of revision. Such revision, however, must be based on an intimate knowledge of a wide range of species and it is doubtful if anyone, certainly not the present writer, as yet possesses the broad and accurate knowledge of species that would be necessary before a complete revision could be proposed. Revision can perhaps best proceed progressively. When groups of species, which show obvious relationship, part of which may now be included in one genus and part in another, come to be thoroughly understood, they should be segregated.

Since very little generic revision has as yet been attempted, it seems best for the present and for the purpose of this contribution to include the species to be described in the genus *Peniophora* interpreted in the generally accepted sense.

Description of Species

Peniophora lauta sp. nov. (Fig. 1)

Fructificatio alba vel glauca, tenuis, pruinosa, sub lente pilosa; basales hyphae paralleliter currentes, non nodoso-septatae; cystidia longa aciculataque 80–120 × 6.5–9 μ, tunicis firmis levibus 0.75–1.5 μ crassis; basidia clavata 20–30 × 7–8 μ, 4 sterigmatibus arcuatis 5–8 μ longis; basidiosporae late ellipsoideae vel subglobosae 6–8 × 5–6 μ, tunicis tenuibus levibusque.

Fructification a white or gray pruinose film spreading over surface of substratum in irregular small patches, pilose under a lens due to the long cystidia, basal hyphae more or less horizontal, few, wall firm, only slightly thickened, without clamps or incrustation; cystidia 80–120 × 6.5–9 μ or more, arising directly from basal hyphae, long, acicular, walls smooth, 0.75–1.5 μ thick, tapering to a slender sometimes mucronate point; basidia arising terminally or laterally on upright branches from basal hyphae, often in clusters, clavate 20–30 × 7–8 μ with four arcuate sterigmata 5–8 μ long; spores broadly ellipsoid to subglobose 6–8 × 5–6 μ flattened on one side, often broadest below the middle, walls thin, smooth, not amyloid.

Specimens examined:

Ontario: On bark of *Thuja occidentalis*, Don Valley near Sunnybrook Park, Toronto, York Co., Sept. 17, 1942, *17581** **type**; woods N.E. of Maple, York Co., Oct. 9, 1943, *18837*; on bark of *Tsuga canadensis*, woods W. of Maple, July 15, 1945, *20120*.

Oregon: On deciduous wood, W. of Corvallis, Benton Co., March 17, 1939, M. Doty *766*, TRT.

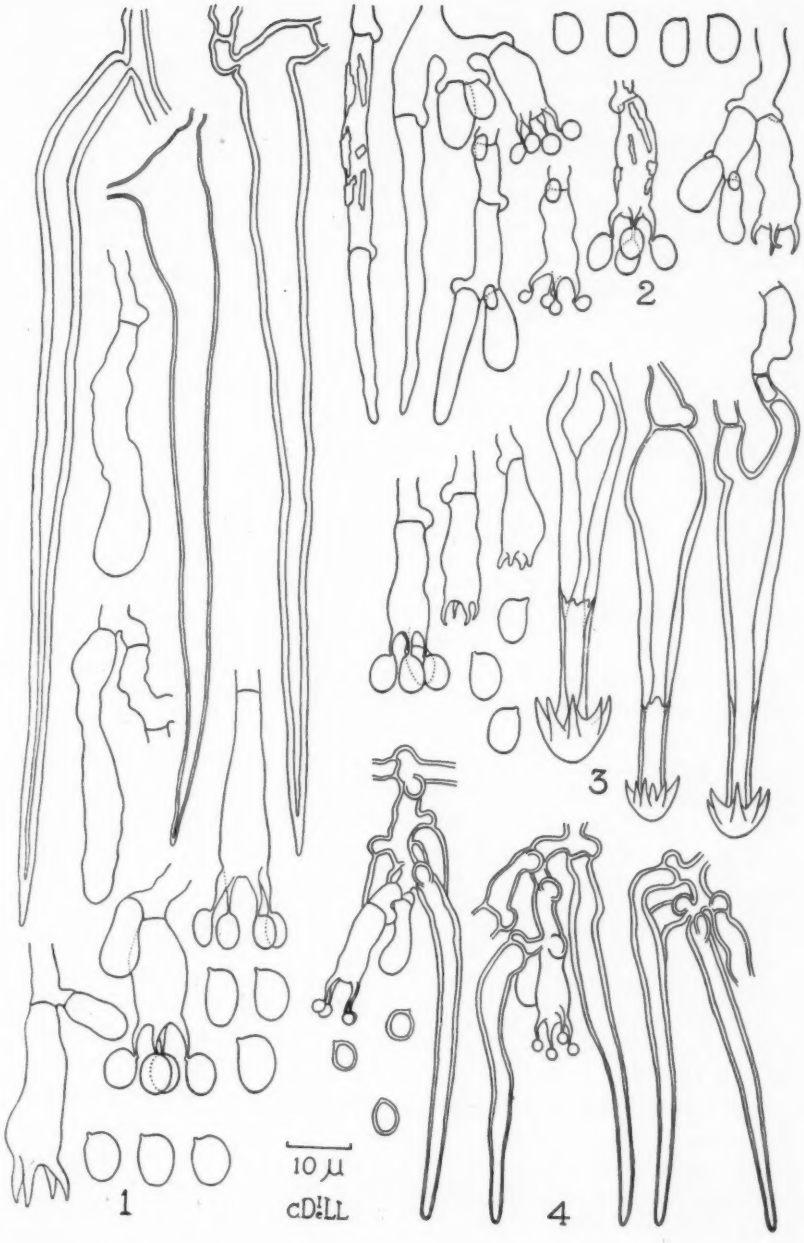
Peniophora lauta would probably have been placed by Bourdot and Galzin (Hyménomycètes de France) in their subsection C *maculiformes* of the Gloeocystidiales. It seems probable that the species, as here described, will be found to include *P. populnea* Bourd. & Galz. The figure and description furnished by them strongly suggest that we are dealing with a form of the same species. *P. populnea* Bourd. & Galz. (1928) is, however, an untenable name, being antedated by the combination *P. populnea* (Peck) Burt (1926). Because of this situation and since we have not seen the type of the Bourdot & Galzin species, it has seemed best to describe our fungus as new rather than to provide a substitute name based on the type of *P. populnea* Bourd. & Galz.

The species is unique because of the subglobose spores and the very long slender cystidia with relatively thin but firm and smooth walls. As listed above, four collections have been available, three on coniferous bark and one on bark and wood of a deciduous tree. All have the essential characters of the species but differ in details of spore size, length and breadth of cystidia, etc. In the drawings for Fig. 1 the two outside cystidia and the upper group of spores are drawn from the type TRT 17581 while the center cystidium and the lower group of spores are drawn from TRT 18837. The third Ontario collection TRT 20120 differs in having the extreme tips of the cystidia mucronate and the spores essentially globoid 6–7 μ in diameter. The Oregon collection also has globoid spores, slightly smaller than in the Ontario collections. The spores are slightly different in each of the four collections but quite uniform in any one. Rather wide variability of the sort indicated is met with most frequently among species that lack clamps and may, perhaps, be correlated with homothallism.

It seems probable that homothallic forms in the Basidiomycetes have been derived from heterothallic ones by occasional haplonts changing in such a manner that the full life and nuclear cycle is carried out on a thallus originating from a single-nucleated spore. This may perhaps be brought about by mutation either in the haplont or the dikaryotic diplont. In any given heterothallic form in which such a tendency developed, a series of 'short

* *The herbarium numbers cited here and following, unless otherwise indicated, are those of the Cryptogamic Herbarium, University of Toronto (TRT). If no collector is indicated the collection was made by the writer.*

FIG. 1. Peniophora lauta; FIG. 2. Peniophora perexigua; FIG. 3. Peniophora hamata; FIG. 4. Peniophora cymosa, *all showing cystidia, basidia, and spores. Drawn to a uniform scale with the aid of camera lucida from potassium hydroxide – phloxine mounts and reproduced at a magnification of approximately 1000×.*


1
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cycled' mutants might originate successively or perhaps intermittently. Originating in this manner there might well be a tendency for variations inherent in the parent heterothallic species to be sorted out and fixed in the resulting series of homozygous lines. In the parent heterozygous form, because of repeated hybridization, such inherent variations would not be so noticeable when several collections are compared.

While it is not suggested that the absence of clamps is proof of homothallism, it is probable that many species lacking clamps are actually homothallic. The above explanation might seem to account for the variation indicated above for *P. lauta*. Other such variable forms lacking clamps include *Pellicularia pruinata* (Bres.) Rogers and *Pellicularia vaga* (Berk. & Curt.) Rogers, both of which show wide variation in spore size and shape. The group of species centering around *Peniophora cremea* (Bres.) Sacc. & Syd. and *P. affinis* Burt also show wide variation and are extremely difficult to evaluate.

Peniophora perexigua sp. nov. (Fig. 2)

Fructificatio hypochnoidea, tenuis, pruinosa, alba; basales hyphae paucae 2.5–4 μ, nodoso-septatae tenuiter tunicatae, crystallis extensis incrustatae; cystidia gracilia acuminata 20–50 $\times$ 2–3.5 μ, in apice e rectis hyphis emergentia; basidia cylindracea vel subclavata 12–17 $\times$ 4.5–5 μ, 4 sterigmata gerentia; basidiosporae late ellipsoideae 6–7 $\times$ 4–5 μ, tunicis tenuibus levibusque.

Fructification hypochnoid, forming thin, pruinose areas on the substratum, white, basal hyphae few, more or less horizontal, 2.5–4 μ in diameter with clamps at all septa, branching at right angles, wall thin, incrusted with elongated crystals; subhymenial hyphae upright also incrusted and with clamps; cystidia slender, acuminate, 20–50 μ $\times$ 2–3.5 μ often incrusted below, may be septate with clamps, arising terminally from upright hyphae, frequently with clusters of basidia at base; basidia often incrusted below, cylindrical or subclavate 12–17 μ $\times$ 4.5–5 μ with four slightly divergent sterigmata 3.5–4.5 μ long; basidiospores broadly ellipsoid 6–7 $\times$ 4–5 μ somewhat flattened on one side with lateral apiculus, walls thin, smooth, non-amyloid.

Specimens examined:

Ontario: On bark of *Tsuga canadensis*, woods W. of Maple, York Co., Sept. 6, 1940, *16529*; Oct. 5, 1940, *16531*, *16532* **type**; Oct. 9, 1943, *18839*; Sept. 8, 1945, *21803*; Brewer Lake, Algonquin Park, R. F. Cain, Sept. 8, 1939, *14166*; Nashville, York Co., R. F. Cain, Oct. 20, 1945, *21801*; Oct. 22, 1945, *21800*; on *Abies balsamea*, Bear Island, Lake Timagami, R. F. Cain, Aug. 1, 1939, *15114*.

This species would appear to be more closely related to *P. pusilla* described below than to any previously described species. It differs, however, in the larger size of the basidia and in the shape and size of the spores. The habitat and gross appearance of the fructifications of the two species are similar, occurring most commonly on bark of hemlock.

Peniophora hamata sp. nov. (Fig. 3)

Fructificatio tenuis, alba, poroso-reticulata vel subpellicularis; subiculum paulum, hyphis non nodoso-septatis; cystidia obclavata 50–75 × 8–12 μ, a convexis et in apice natis 7–8 ×8.5 μ pileis qui in margine 10–12 decurvatis furcis 3–4 μ longis praediti sunt praepilata; basidia clavata cylindracea 16–20 × 6–7.5 μ, 4 sterigmatibus 3.5–4 μ longis; basidiosporae late ellipsoideae 5.5–7.5 × 4–4.5 μ, tunicis tenuibus levibusque.

Fructification white, thin, broadly effused with indeterminate margin, porous reticulate to subpelliculose, separable; subiculum scanty with clamped hyphae 2.5–3 μ broad, indistinct; cystidia obclavate, 50–75 μ long, 8–12 μ broad ¼ distance from base, 3.5–4.5 μ, ¼ distance from apex, capitate with convex terminal caps 7.5–8.5 μ broad bearing 10–12 marginal decurved prongs 3–4 μ long; walls somewhat thickened especially in middle portion, this region swelling in potassium hydroxide, finally dissolving completely, terminal third and caps with thick walls not affected by potassium hydroxide; basidia clavate-cylindrical 16–20 × 6–7.5 μ bearing four slender slightly arcuate sterigmata 3.5–4 μ long; basidiospores broadly ellipsoid 5.5–7.5 × 4–4.5 μ, flattened on one side with prominent lateral apiculus, walls thin, smooth, non-amyloid.

Specimens examined:

Ontario: On coniferous wood, usually *Abies balsamea*. Bear Island, Lake Timagami, July 18, 1936, *10462*; Aug. 8, 1936, *9866*; Aug. 12, 1936, *9872*; R. Biggs, July 17, 1936, *12488*; July 21, 1936, *10818*; Aug. 4, 1936, *9873*; Aug. 6, 1936, *12483*; Aug. 12, 1936, *9867*, *9877*; Aug. 19, 1936, *9848*; R. F. Cain, Aug. 1, 1939, *15120*, *15239*; Gull Lake Portage, Lake Timagami, Aug. 10, 1936, *9946*; Long Point, East Mainland, Lake Timagami, R. Biggs, Sept. 12, 1936, *9871* **type**; Gomphidius Bay, West Mainland, Lake Timagami, R. Biggs, Sept. 1, 1936, *9876*; Costello Lake, Algonquin Park, R. F. Cain, Sept. 4, 1939, *14565*, *14569* (on *Thuja occidentalis*); trail to Pine Tree Lake, Algonquin Park, Sept. 16, 1939, *14397*.

The species described above is a member of the third xxx division of the section Tubuliferae of the Bourdot and Galzin classification. This division includes for the most part species with thick-walled cystidia, often with capillary lumen at the base, the walls of which are more or less affected by potassium hydroxide, often dissolving completely.

P. hamata is a unique member of this group because of the cystidial cap with downward projecting prongs. This cap is apparently of different composition and is not affected by potassium hydroxide while the lower part of the wall of the cystidium swells greatly in potassium hydroxide, finally dissolving more or less completely. The only other member of this section familiar to us, having an apical por. .n of the cystidium not affected by potassium hydroxide is *Peniophora inornata* described in this paper.

Peniophora cymosa Rogers & Jackson sp. nov. (Fig. 4)

Fructificatio tenuis, sicca minute pilosa, alutacea; hyphae plerumque crasse tunicatae, nodoso-septatae, contortae, 3–4.5–7.5 μ diam., altitudinibus variis vel basidium vel cystidium gignentes; cystidia subulata, acutata, ubique crasse tunicata, 50–78 × 4–6 μ; basidia cylindracea, 10–12 × 4.5–5.5 μ, sterigmata quattuor gracilia, curvula, divergentia, 3.5 μ long. gerentia, tunica inferiori subincrassata persistentique; sporae globosae, crasse tunicatae, uniguttulatae, 4.5–5 μ diam.

Fructification thin, adnate, when fresh probably gelatinous-waxy, when dry buffy, discontinuous, minutely pilose; composed of distinct hyphal branches growing out perpendicular to the substratum and giving rise at brief intervals to cystidia and basidia—or better, repeatedly terminating in either a basidium or a cystidium, and repeatedly continued by acropetal growth of a lateral branch; hyphae short-celled with prominent clamps, mostly thick-walled, subarticulate, 3–4.5–7.5 μ, the fertile branches sinuose and retaining fragments of the old discharged basidia still attached; cystidia slender-subulate, 50–78 $\times$ 4–6 μ, often somewhat curved, thick-walled throughout, acute, sometimes with a resinous droplet at the tip, arising at various levels from the fertile hyphae; basidia subcylindric, about 10–12 $\times$ 4.5–5.5 μ, arising at various levels, the lower wall thickened and persistent, bearing four slender, curved, divergent sterigmata 3.5 μ long; spores subglobose, 4–5 μ in diameter, slightly flattened beyond the apiculus, with appreciably thickened walls, smooth, guttulate, non-amyloid.

Specimens examined:

Ontario: On partly decayed coniferous wood, Bear Island, Lake Timagami, Aug. 19, 1936, R. Biggs, *10837*, *10785*.

North Carolina: Highlands, Aug. 17, 1933 (first foray), G. W. Martin, *1321*, **type**, in TRT and IA.

Peniophora cymosa would perhaps fall in the section Hyphales of the Bourdot and Galzin classification. This section, however, as treated by them, includes a rather heterogeneous assortment of obviously unrelated forms none of which seem close to *P. cymosa*.

The unique features of the species here described are the thick-walled subglobose spores, and the uniformly thick-walled unincrusted cystidia, which are often more acutely pointed than brought out in the drawings (Fig. 4).

Peniophora probata sp. nov. (Fig. 5)

Fructificatio late effusa, alba, dein isabellina, 75–100 μ crassa; subiculum laxe intextis hyphis nodoso-septatis compositum; hyphae inferiores irregularibus e cellulis 8.5 μ diam. brevibus; hyphae rectae et supra paralleles, 2.5–3.5 μ diam., nodulosae; cystidia clavata vel ventricoso-clavata, 55–70 $\times$ 8–13 μ, tunicis inferioribus tenuibus, parum incrassatis, apicem versus curvulis; basidia gracilia clavata 20–30 $\times$ 4–4.5 μ, 4 sterigmatibus 3.5–5 μ longis; basidiosporae ellipsoideae 4.5–6 $\times$ 2.5–3 μ, tunicis tenuibus levibusque.

Fructification white when young, becoming ochraceous to isabelline, broadly effused, separable, 75–100 μ thick, hymenium becoming subceraceous; subiculum of loosely interwoven hyphae with clamps, upright and parallel in upper half 2.5–3.5 μ in diameter, made up of short cells appearing nodulose due to prominent clamps, becoming broader below and connecting with a

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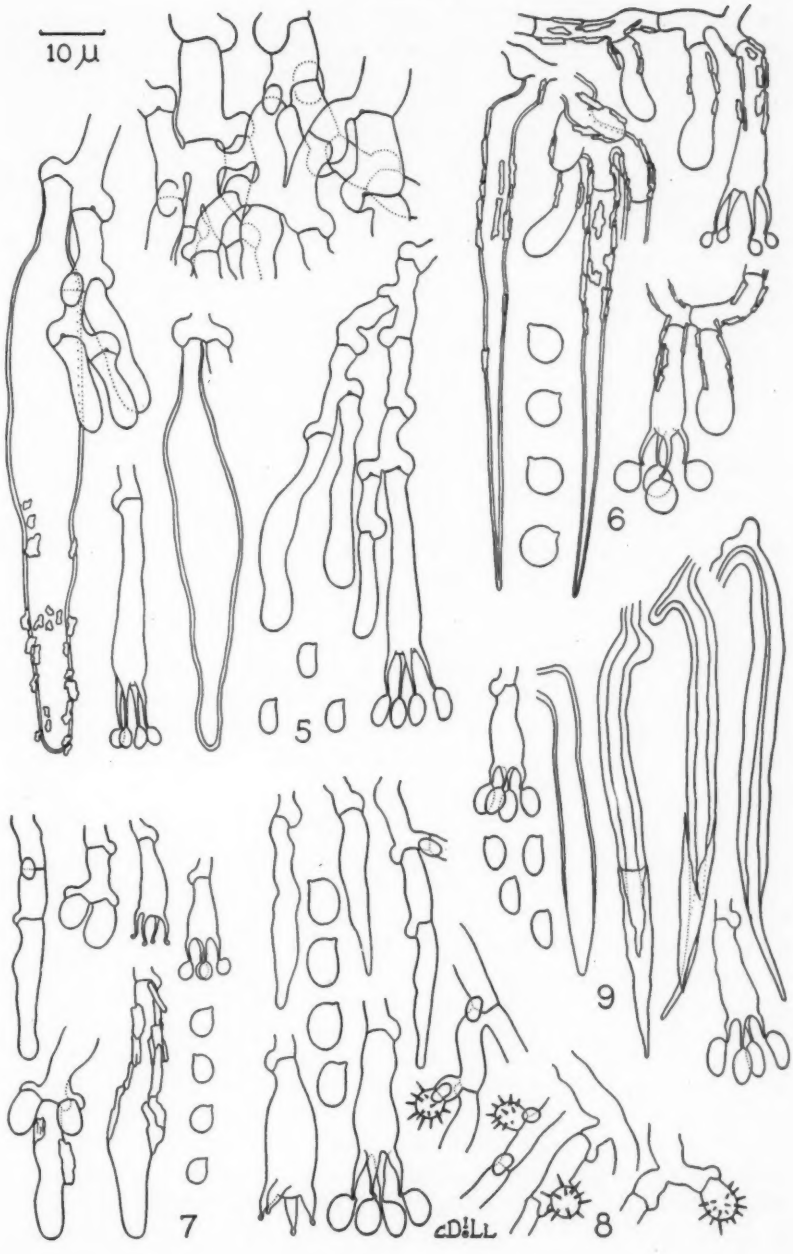
FIG. 5. Peniophora probata; FIG. 6. Peniophora ralla; FIG. 7. Peniophora pusilla; FIG. 8. Peniophora compta; FIG. 9. Peniophora inornata, *all showing cystidia, basidia, and spores. In Fig. 5 above, a group of wide subicular hyphae and in Fig. 8 at the right a group of aculeate bodies on the subicular hyphae. All figures drawn to a uniform scale with the aid of a camera lucida from potassium hydroxide – phloxine mounts and reproduced at an approximate magnification of 1000×.*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looser subiculum made up of intertwined hyphae of irregular short cells varying in diameter to 8.5 μ; cystidia clavate or ventricose clavate, 55–70 × 8–13 μ, wall thin or becoming slightly thickened in upper half or third, rounded and often incrusted with crystals at apex; basidia slender clavate 20–30 × 4–4.5 μ extended slightly above level of developing basidia when mature, with four slender upright or slightly divergent sterigmata 3.5–5 μ long; spores ellipsoid, 4.5–6 × 2.5–3 μ, flattened and appearing straight on one side with lateral apiculus, walls thin, smooth, non-amyloid.

Specimens examined:

Ontario: On decaying wood of *Pinus*, Bear Island, Lake Timagami, July 15, 1937, *13708*; Aug. 5, 1937, *13709*; July 22, 1935, *8905*; Aug. 13, 1938, *13989*; July 15, 1938, *13996*; Aug. 12, 1943, *19090*; Aug. 7, 1943, *18832*, **type**; R. Biggs, Aug. 14, 1936, *12903*; R. F. Cain, Sept. 7, 1935, *8663*, July 19, 1939, *15288*; on wood of *Tsuga canadensis*, woods W. of Maple, York Co., July 15, 1945, *20119*.

According to the treatment of Bourdot & Galzin, this species would perhaps be included in the subsection A *submembranaceae* of their section Gloeocystidiales. It is however not a typical member of that group if such species as *P. argillacea* (Bres.) Sacc. & Syd., *P. medioburiensis* Burt, *P. clavigera* (Bres.) Bourd. & Galz., and *P. fusca* Burt represent the more typical forms. Our species may be separated from others in the genus by the combination of cystidial and spore characters taken together with the presence in the subiculum of broad hyphae made up of short, somewhat irregular cells as illustrated in the upper portion of Fig. 5. When crushed mounts are made in potassium hydroxide – phloxine, there is evident a considerable amount of oily material.

Peniophora ralla sp. nov. (Fig. 6)

Fructificatio alba, delicata, pruinosa; hyphae subiculi paucae, 3.5–4 μ diam., plerumque incrustatae, non nodoso-septatae; cystidia terminalia vel e basalibus hyphis emergentia, aciculata 60–75 × 4–5.5 μ, ad apicem acutatum attenuata, tunicis firmis leviterque incrassatis; basidia clavata vel subcylindracea, 16–20 × 4–5 μ, 4 sterigmata arcuata 5–6.5 μ longa gerentia; basidiosporae globosae, 5–6 μ diam., tunicis tenuibus levibusque.

Fructification white, delicate pruinose, interrupted; basal hyphae few, usually incrusted, 3.5–4 μ in diameter, without clamps, wall thin but often appearing to be thickened because of incrustation; cystidia terminal or arising as upright branches from basal hyphae, acicular 60–75 × 4–5.5 μ at base, tapering to a fine point, wall firm, slightly thickened, appearing rugose toward base due to fine incrustation, with simple septum close to point of origin; basidia clavate to subcylindrical, 16–20 × 4–5 μ with four arcuate sterigmata 5–6.5 μ long, often incrusted at base, arising directly as side branches of basal hyphae or in clusters; basidiospores globoid, 5–6 μ in diameter, not appreciably depressed at one side, with prominent apiculus; walls thin, smooth, non-amyloid.

Specimens examined:

Ontario: On bark and wood of *Abies balsamea*, portage to Gull Lake, Lake Timagami, Aug. 10, 1936, *9770*, *10789*; R. Biggs, Aug. 10, 1936, *9771*, **type**; Sept. 9, 1936, *10790*.

Peniophora ralla might be compared to the forms included by Bourdot & Galzin in the second xx group of their section Tubuliferae as for example *P. clematidis* Bourd. & Galz. and *P. abietis* Bourd. & Galz. In both these species, however, the cystidia have very much thickened walls, rugose on the surface, and clamps are present in the hyphae. It is perhaps more closely related to *P. lauta*, described above, differing however in the shorter and more slender cystidia and in spore characters.

Peniophora pusilla sp. nov. (Fig. 7)

Fructificatio alba, divergens, pruinosa; subiculum infra laxis subparalleliter currentibus hyphis, 2.5–4 μ diam., nodoso-septatis, supra cymoso modo ramos emittentibus, floccosis crystallis crasse incrustatis; cystidia cylindracea vel clavata, in apice obtusa, 25–45 × 4.5–6.5 μ, tenuiter tunicata; basidia cylindracea vel clavata, 6.5–10× 4–4.5 μ, 4 gracilia 3–4.5 μ longa sterigmata gerentia; basidiosporae subglobosae 3.5–4.5 × 3–3.5 μ, tunicis tenuibus levibusque.

Fructification white, forming a pruinose spreading growth on substratum usually not forming a continuous hymenium, margin indeterminate; subiculum formed of loose hyphae subhorizontal below, branching in a cymose manner above, lower hyphae with slightly thickened walls, 2.5–4 μ broad, occasionally inflated, clamps present, subhymenial hyphae thinner, heavily incrusted with flaky highly refractive crystals not readily soluble in potassium hydroxide that may extend to base of basidia and cystidia; cystidia cylindrical or clavate, arising from clusters of basidia, with thin walls, obtuse at apex, 25–45 × 4.5–6.5 μ often with septum and clamp below center, emergent by about half their length; basidia cylindrical or clavate 6.5–10 × 4–4.5 μ with four slender divergent sterigmata 3–4.5 μ long; basidiospores subglobose 3.5–4.5 × 3–3.5 μ, depressed on one side, with minute obliquely placed apiculus, walls thin, smooth, non-amyloid.

Specimens examined, all on bark of *Tsuga canadensis*:

Ontario: Brewer Lake, Algonquin Park, R. F. Cain, Sept. 5, 1939, *14539*.

Quebec: Burnet, M. Timonin, Oct. 1, 1935, OTB, F*6785*, **type**, TRT.

New Hampshire: Lac Lincoln, H. G. Eno, Sept. 27, 1939, BPI, FP *84884*, TRT.

As previously noted under *P. perexigua*, this species is perhaps closely related. The basidia are however only about half the length of those in *P. perexigua* and the spores are much smaller and of different shape. Both species have a similar type of incrustation on the hyphae, which may be correlated with the similar substratum. No difficulty is encountered in separating the two from the microscopic characters.

Peniophora compta sp. nov. (Fig. 8)

Fructificatio tenuis, divergens, alba vel cremea, subpellicularis; subiculum ex hyphis laxe intricatis, 3.5–5.5 μ diam., nodoso-septatis, tunicis firmis partimque leviter incrassatis praeditis, ad basim vel ad apicem structuras quae globosae vel subglobosae cellulae 3.5–5.5 μ diam. sunt hic et illic ferentibus compositum, tunicis tenuibus parce distributis aculeatis spinis 0.75–1.5 μ longis praeditis; cystidia gracilia, acuminata 25–45 × 2.5–4.5 μ, tunicis tenuibus interdum septatis; basidia clavata vel subcylindracea 15–25 × 6.5–8.5 μ, 4 sterigmata 4.5 μ longa gerentia; basidiosporae late ellipsoideae 5.5–8 × 3.5–5 μ, tunicis tenuibus levibusque.

Fructification thin spreading, whitish or cream, subpelliculose, margin indeterminate, separable and friable when dry; hyphae of subiculum loosely arranged, with regular clamps, walls firm, in part slightly thickened, of varying diameter, 3.5–5.5 μ, bearing occasional aculeate bodies consisting of a globose or subglobose cell 3.5–5.5 μ in diameter, sessile or terminal on short branch, always subtended by a clamp, wall thin with sparsely distributed aculeate spines 0.75–1.5 μ long; cystidia (or cystidioles) slender, acuminate, 25–45 × 2.5–4.5 μ, somewhat flexuous, arising in the hymenium or subhymenium and extending beyond basidia for $\frac{1}{3}$ – $\frac{1}{2}$ length, with septum and clamp at base or occasionally also at $\frac{1}{3}$ – $\frac{1}{2}$ from base to apex, walls thin not incrusted; basidia clavate or subcylindrical 15–25 × 6.5–8.5 μ bearing four straight or slightly divergent sterigmata 4.5 μ long; basidiospores broadly ellipsoid, 5.5–8 × 3.5–5 μ slightly flattened and appearing nearly straight on one side, with lateral apiculus, walls thin, smooth, non-amyloid.

Specimens examined:

Ontario: On decayed coniferous wood, frequently *Pinus Strobus*. Bear Island, Lake Timagami, July 21, 1939, *15019* **type**; Aug. 18, 1944, *19742*, *20030*; Paradis' Bay, Lake Timagami, Aug. 22, 1935, R. Biggs, *8746*; Aug. 26, 1936, *16675*; woods S. of Aurora, York Co., Oct. 16, 1938, *13717*, *13718*.

The cystidia in this species are of the type that might be referred to as cystidioles and the species could perhaps as well be included in *Corticium* as in *Peniophora*.

In morphological details of cystidia and spores, as indicated by the drawings (Fig. 8), this species might appear to be close to *P. perexigua* (Fig. 2), but in gross appearance the two are quite different. In *P. compta* the cystidia arise in the hymenium perhaps as modified basidia, while in *P. perexigua* they arise from the subiculum.

The unique feature of *P. compta* is the presence of the characteristic aculeate bodies on the hyphae of the subiculum. So far as we are aware no structure of this sort has been previously described in any thelephoraceous resupinate. These structures are certainly not of the same nature as the caliciform bodies that are described for *P. caliciferum* by Litschauer (Oesterr. Botan. Z. 77 : 126. Fig. 4. 1928) and that also occur in other forms of the *P. tenuis* (Pat.) Massee complex. There is no suggestion that they are disseminated and they do not appear to be chlamydospores. Their function is obscure.

Peniophora inornata Jackson & Rogers sp. nov. (Fig. 9)

Fructificatio tenuis, alba, pruinoso, sub lente hispida; hyphae subiculi plurimum paralleliter currentes, 3–3.5 μ diam., tunicis interdum leviter incrassatis, nodoso-septatae; cystidia infra cylindracea, ad acutatum apicem attenuata, pileo acuto tecta, 50–75 × 4–5.5–7 μ, tunicis angusto lumine, infra crassis, supra tenuibus, in KOH praeter pileum acutum dissolubilibus; basidia cylindracea, 10–16.5 × 4.5–6 μ, 4 sterigmata gerentia; basidiosporae ellipsoideae 4.5–5.5–6 × 2.5–3.5 μ; tunicis tenuibus levibusque.

Fructification a thin, white, delicate pruinose film separating on drying to form a fine reticulum, hispid under a lens from the numerous cystidia; hyphae of subiculum somewhat obscure, mostly horizontal, 3–3.5 μ in diameter, in part with slightly thickened walls and with clamps at septa; cystidia arising from upright or from basal hyphae, 50–75 × 4–5.5–7 μ, cylindrical below, tapering above to an acute apex that is sheathed by an acutely pointed cap; wall thick below with narrow lumen, thin above, soluble in potassium hydroxide, except for the cap of different composition; basidia cylindrical 10–16.5 × 4.5–6 μ with four sterigmata; basidiospores ellipsoid, 4.5–5.5–6 × 2.5–3.5 μ, somewhat flattened and appearing straight on one side with lateral apiculus, walls thin, smooth, non-amyloid.

Specimens examined:

Ontario: On coniferous wood, usually *Abies balsamea* or *Pinus Strobus*. Bear Island, Lake Timagami, Aug. 8, 1944, *20011*; Aug. 16, 1945, *21804*; Aug. 21, 1944, *20032*; Aug. 17, 1943, *19091*; Paradis' Bay, Lake Timagami, R. Biggs, Aug. 17, 1936, *10838*, *10839*; Timagami Island, Lake Timagami, R. F. Cain, July 21, 1937, *13720*; portage to Spawning Lake from Lake Timagami, Aug. 11, 1939, *15004* **type**; Opeongo Lake, Algonquin Park, R. F. Cain, Sept. 18, 1939, *14899*.

Oregon: On *Pinus contorta*, Sutton Lake, Nov. 26, 1937, A.M. and D. P. Rogers, *424* also TRT; on *Pseudotsuga taxifolia*, Chetco River, above Brookings, July 12, 1939, A.M. and D. P. Rogers *764* also TRT.

The species here described is another member of the third xxx division of Bourdot & Galzin's section Tubuliferae. It is distinguished from the other members of the group primarily because of the acute caps over the tips of the cystidia. This cystidial cap, as in *P. hamata*, is of different composition from the thickened wall of the cystidium below. At first glance the thickened cap appears to blend insensibly into the wall below but when treated with potassium hydroxide the lower wall dissolves completely while the acutely pointed caps remain unaffected. These caps appear to develop independently of the main wall of the cystidium as the latter matures. Young cystidia show no signs of a cap and occasionally a nearly mature cystidium is found without a cap, in which case the upper part of the cystidium wall is shown to be quite thin and developed to an acute apex as shown in the cystidium farthest to the left in Fig. 9. It seems possible that these caps are formed as a secretion from the wall.

This species was recognized independently as new by Dr. D. P. Rogers from collections made in Oregon and I am indebted to him for the specimens from that state recorded above.

A HOMOTHALLIC, SHORT-CYCLE STRAIN OF *UROMYCES POLYGONI* (PERS.) FUCKEL[1]

By A. M. Brown[2]

Abstract

A strain of *Uromyces Polygoni* (Pers.) Fuckel collected on *Polygonum aviculare* L. at Winnipeg was found to be homothallic. Attempts to infect *P. erectum* L. and *P. neglectum* Besser with sporidia, aeciospores, and urediospores of this rust were unsuccessful. Of the sporidial infections on *P. aviculare*, whether originating from a single sporidium or from two or more sporidia, most produced aecia only, but some produced uredia only, and a few produced aecia and uredia in association. Pycnia were not visible in any of the sporidial infections when examined with a hand lens but, in microtome sections cut from infections that had developed aecia, a few pycnia containing pycniospores were observed. No flexuous hyphae and periphyses were seen in these pycnia.

Introduction

Uromyces Polygoni (Pers.) Fuckel occurs yearly in Western Canada and commonly inhabits species of *Polygonum* (1, 2, 4). At Winnipeg, Man., in the summer of 1942, and again in 1943, this rust was observed on *Polygonum aviculare* L. but not on *P. erectum* L. or *P. neglectum* Besser, although the three species grew in close proximity to each other. These observations suggested that a strain of *U. Polygoni* having a restricted host range was present in the neighborhood of Winnipeg. To ascertain the validity of this assumption, infection experiments were undertaken. These had as a further objective the determination of the sexual behavior of this rust.

Materials and Methods

Telial material of *U. Polygoni* was collected from *P. aviculare* in October, 1943. It was kept in paper bags in the laboratory at ordinary room temperature during the ensuing winter months. In April, 1944, teliospore germination was induced by subjecting selected small portions of the telial material to alternate wetting and drying. During the months of May and June, seedling plants of *P. aviculare*, *P. erectum*, and *P. neglectum* were enclosed in moist chambers and germinating teliospores were suspended over them, in a manner similar to that described by Craigie (3).

Experimental Results

From 12 to 14 days after the plants had been exposed to sporidial showers, aecia or uredia, and in some cases both aecia and uredia, developed on *P. aviculare*, but no rust developed on *P. erectum* or *P. neglectum*.

[1] *Manuscript received January 5, 1948.*

Contribution No. 924 from the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa.

[2] *Assistant Plant Pathologist, Dominion Laboratory of Plant Pathology, Winnipeg, Man.*

Although many isolated and compound infections occurred on *P. aviculare*, which proved to be a congenial host, in none of them could pycnia or pycnial nectar be seen, even with the aid of a hand lens. In microtome sections cut from monosporidial infections that had developed aecia, a few pycnia were observed. Pycniospores were present at the ostioles of a few of these pycnia but flexuous hyphae and periphyses were absent. Sporidial infections that had produced uredia only, or aecia and uredia in association, were not examined in microtome sections to determine the presence or absence of pycnia.

To obviate the chance transfer of pycniospores by insects from one infection to another, all of the infected plants were enclosed in wire-screen cages.

Altogether 110 well isolated, and apparently monosporidial infections, were observed and, of these, 96 produced aecia spontaneously when they were from 12 to 14 days old. Of the others, nine infections produced uredia but no aecia, and five produced both uredia and aecia.

As insects had been excluded from the infected plants and as no attempt was made to transfer pycniospores from one infection to another, the development of aecia and uredia in these monosporidial infections apparently resulted from nuclei contained within the individual thalli. Even if the few pycniospores observed in some infections had been fortuitously transferred from one infection to another, it is doubtful if, in the absence of flexuous (receptive) hyphae, their intervention would have been effective.

In addition to the infections just mentioned, 20 compound infections, that is, infections originating from two or more sporidia, were selected for observation. Of these infections, 14 produced aecia simultaneously in both components, two produced uredia in both components, and four produced aecia in one component and uredia in the other.

Examination of microtome sections cut from monosporidial infections indicated that the binucleate phase appeared first in the aecial primordia. Some incipient aeciospores developing from primordial aecial cells were observed to be uninucleate, although most were binucleate.

As previously mentioned, this rust appeared under natural conditions to be limited to *P. aviculare*. The problem of its host range was further investigated by sowing aeciospores and urediospores separately on well established, rust-free plants of *P. aviculare*, *P. erectum*, and *P. neglectum*. The inoculations were repeated a number of times with identical results: rust developed only on *P. aviculare*.

Conclusions

The evidence just presented shows that the strain of *U. Polygoni* investigated has a restricted host range. Its sporidia, aeciospores, and urediospores, although repeatedly sown on *P. aviculare*, *P. erectum*, and *P. neglectum*, caused infection of the first-named species only.

Isolated monosporidial infections of *U. Polygoni* produced aecia spontaneously without the intervention of pycniospores. Some infections produced

uredia instead of aecia, and a few produced both aecia and uredia simultaneously and in association, presumably on the same mycelium. Evidently the rust mycelium present in each of these infections contained within itself the nuclear elements necessary to initiate the conjugate nuclear condition. On the basis of this evidence it is concluded that this strain of *U. Polygoni* is homothallic.

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STUDIES OF CANADIAN THELEPHORACEAE

II. SOME NEW SPECIES OF *CORTICIUM*[1]

By H. S. Jackson[2]

Abstract

In the following pages nine species of *Corticium*, found in Ontario and presumed to be new to science, are described and illustrated. These were encountered in connection with a general taxonomic study of resupinate Thelephoraceae. In addition, a redescription and discussion of a species recently published from Europe, also found to be common in Ontario, is included.

Introduction

The general remarks included in the introduction of the first number of this series (See Footnote 1) will for the most part be applicable to the present contribution as well, except where they refer specifically to the genus *Peniophora*.

As was true of the first, this second paper is the result of a general study of the resupinate Thelephoraceae of North America, with special reference to those found in Canada, during which a large number of specimens have been examined. In the pages that follow, nine species falling in the genus *Corticium*, which to the best of our knowledge are new to science, are described, illustrated, and discussed. In addition a redescription of *Corticium sulfureo-isabellinum* Litschauer, which was described in a paper by Pilát (4) published in 1940 in Europe and which has been found to be common in Ontario, is included with detailed discussion.

The genus *Corticium* as usually delimited includes a large assemblage of simple resupinate species having no special inclusions other than gloeocystidia or cystidioles, and usually having colorless spores. Recent European students of the group have for the most part segregated those species possessing gloeocystidia in the genus *Gloeocystidium* H. & L. American students, on the other hand, have included the gloeocystidiate species in *Corticium* on the grounds that to segregate such forms on the basis of a single character merely results in another collective genus composed of species or groups of species that are unrelated. In any case *Gloeocystidium* H. & L. is an untenable name, being antedated by *Gloeocystidium* Karst. *Gloeocystidiellum* Donk, with *Corticium*

[1] *Manuscript received January 9, 1948.*

Contribution from the Department of Botany, University of Toronto, Toronto, Ont. This study was carried out with the assistance of a grant in aid of research furnished by the University of Toronto.

No. I of this series, "Some New Species of Peniophora", was published in Can. J. Research, C, 26 : 128-139. 1948.

[2] *Professor of Mycology and Head, Department of Botany.*

The writer is indebted to Miss Margaret H. Thomson for the preparation of the Latin diagnoses, to Miss Charlotte M. Dill for the preparation of the drawings, and to Miss E. Ruth Dearden for assistance in the preparation of the technical descriptions. Dr. D. P. Rogers has been kind enough to read the manuscript.

porosum B. & C. as its type, is an available generic name for the gloeocystidiate forms. This generic name, because of its type, should perhaps be used, if at all, only in a restricted sense to include certain of the species having amyloid spore walls.

Since *Corticium*, like *Peniophora*, is obviously a collective genus, including a heterogeneous assemblage of unrelated groups of species it seems best for the present to retain the gloeocystidiate forms therein, until the relationships of such forms are better understood. In the present contribution two such species are included.

As was noted in the first paper of this series the resupinate Homobasidiomycetes are greatly in need of revision. The genus *Corticium* is no exception. Species or groups of species now included in this assemblage include the basic members of several natural evolutionary lines, some of which will undoubtedly be found to include species now in other genera of the Thelephoraceae, as well as species now included in various genera of the resupinate Hydnaceae and in the genera *Merulius* and *Poria* of the older concept of the Polyporaceae.

For the future one can envision a classification of the simpler Homobasidiomycetes based upon that first proposed by Patouillard (3), which has been elaborated by Bourdot and Galzin (1) and more recently by Donk (2). In this classification, the old family name Thelephoraceae will probably disappear and its place be taken by a series of families grouped under the ordinal name Aphyllophorales, some of the families perhaps divided into subfamilies and tribes. The basis of one or more of these families, subfamilies or tribes will emerge from a regrouping of species now or formerly in *Corticium* combined with species from other genera or families.

Recent segregates, illustrative of this trend in classification and having their basic species formerly in *Corticium*, which appear to be acceptable, include *Ceratobasidium* Rogers (5) and the related genus *Pellicularia* Cooke (=*Botryobasidium* Donk) (Rogers (6)), and *Trechispora* Karsten as revised and reviewed by Rogers (7). In *Pellicularia*, species formerly in *Corticium* and *Peniophora* are included. In *Trechispora* species formerly in *Corticium* are combined with others formerly in the genus *Grandinia* of the Hydnaceae and in *Poria* of the Polyporaceae. The closely related genus *Sistotrema* also formerly in the Hydnaceae includes properly only one or two pileate species. In all the segregates mentioned, the basic character considered of phylogenetic significance centers in the type of basidium.

Other groups of related species in *Corticium* can be more or less clearly recognized. Most of the species in the section Humicola of the Bourdot & Galzin classification form a natural group when combined with such species as *Sistotrema variecolor* Bourd. & Galz. and certain of the rough spored species of *Poria* such as *P. candidissima* (Schw.) Cooke. For this group the generic name *Phlebiella* Karsten, with *Corticium sulphureum* (Pers. ex Fries) Fries (= *Hypochnus fumosus* sensu Burt) as the type, is apparently available (Rogers (7, p. 79)). Some or all of the species of *Phlebia* when compared

with certain ceraceous species now in *Corticium* and *Peniophora* will probably be found to form a natural assemblage. Gloeocystidiate species such as *Corticium* (*Gloeocystidium*) *pallidum* Bres. when combined with the more characteristic members of Bourdot and Galzin's section Gloeocystidiales of *Peniophora* form a natural group. Certain species of *Corticium* will undoubtedly be found to have their nearest relatives among colorless-spored species of *Merulius*. Other examples might be cited but the cases mentioned are sufficient for the present discussion.

As such groups come to be more clearly understood and their limits established they should be segregated. As segregation proceeds the relationships of the remaining species should become more clear. Fortunately, students of the group are so far proceeding slowly and cautiously in segregating such groups. A major problem, which must be basic to any sound revision, is to determine what characters are of phylogenetic significance and reflect real relationships.

While the writer is in full sympathy with the necessity for revision, it seems undesirable at present to describe species and assign them to available genera until such genera are covered by a monographic or at least a synoptical treatment. For this reason the species described below are all assigned to *Corticium*. As revision proceeds, a proper place will be found for them in other genera.

Description of Species

Corticium delectabile sp. nov. (Fig. 1)

Fructificatio effusa, cremea vel citrula, subceracea, 140–150 μ crassa; basales hyphae subiculi paucae, fere rectae, non nodoso-septatae, 3.5–4 μ diam., tunicis tenuibus, hyalinis; gloeocystidia nulla; basidia magna, 50–80 × 7–9 μ, cylindracea vel subclavata, flexuosa, interdum subventricosa, 3 vel 4, raro 2 vel 5 arcuata subulataque sterigmata gerentia; basidiosporae subglobosae vel lacrimiformes 7–9 × 6–8 μ, tunicis tenuibus, hyalinis, levibus.

Fructification effused, cream to yellow, subceraceous, adnate, 140–150 μ thick, margin indeterminate, horizontal basal subicular hyphae few, hyphae for the most part upright, without clamps, 3.5–4 μ in diameter, branching often pseudo-dichotomously, wall thin, colorless; gloeocystidia none; basidia cylindrical to subclavate, flexuous and occasionally subventricose, 50–80 × 7–9 μ, bearing three or four, rarely two or five arcuate, subulate sterigmata 7–8.5 μ long; basidiospores in side view subglobose, laterally depressed with prominent apiculus, in face view subglobose to lacrimiform, 7–9 × 6-8 μ, walls thin, hyaline, smooth, non-amyloid.

Specimens examined:

Ontario: On partly decayed, decorticated deciduous wood, woods W. of Maple, York Co., R. F. Cain, Oct. 1, 1938, *13684** **type**; on humus, woods S. of Aurora, York Co., Oct. 10, 1937, *16722*; on ground in woods, Don Valley, Toronto, York Co., R. F. Cain, Sept. 22, 1934, *8672*; woods W. of Maple, Sept. 22, 1940, *16432*.

* *The herbarium numbers cited here and following, unless otherwise indicated, are those of the Cryptogamic Herbarium of the University of Toronto (TRT). If no collector is named the collection was made by the writer.*

The relationship of this species is not clear. It has some resemblance to *C. Macounii* Burt but differs in essential respects. In the latter species clamps occur at the septa, gloeocystidia and hyphal paraphyses are present, and the basidia are typically two-spored.

Gloeocystidia seem to be absent in *C. delectabile* though young basidia might easily be mistaken for them. The basidia are long, narrow, and flexuous, somewhat resembling those in some species of *Aleurodiscus*, but the species seems more properly referred to *Corticium*. The unique features are the long basidia and the characteristic spores.

Corticium electum sp. nov. (Fig. 2)

Fructificatio tenuis, 50–75 μ, late effusa, alba vel eburneo-citrula, viva cereo-ceracea, sicca pruinosa vel farinosa, deinde continua, membranacea, levis vel minute rimosa; subiculum ex hyphis laxe intricatis, saepe ramosis nodulosisque 1.5–3 μ diam. nodoso-septatis compositum; gloeocystidia nulla; basidia gracilia cylindracea, 20–30 × 2.5–3.5 μ, infra subventricosa, maturitate supra hymenium emergentia, 4 gracilia leviter divergentia sterigmata 4.5 μ longa ferentia; basidiosporae late reniformes 3.5–4 × 2–2.5 μ, tunicis tenuibus, subtiliter echinulatis, in solutione KOH levibus, valde amyloideis.

Fructification 50–75 μ thick, becoming widely effused on decorticated wood, white or ivory yellow to pinkish buff, when fresh waxy ceraceous, when dry pruinose to farinose, becoming continuous, membranous, smooth or minutely rimose; subiculum of more or less upright loosely interwoven, frequently branched nodulose hyphae, with clamps, 1.5–2 μ in diam., becoming collapsed and indistinct; typical gloeocystidia absent; basidia slender cylindrical, 20–30 × 2.5–3.5 μ, somewhat ventricose and broader below, extruded beyond the level of the hymenium at maturity, bearing four slender straight or slightly divergent sterigmata 4.5 μ long; basidiospores broadly reniform 3.5–4 × 2–2.5 μ with inconspicuous lateral apiculus, walls thin, delicately and closely echinulate, strongly amyloid in Melzer's solution, becoming smooth in potassium hydroxide.

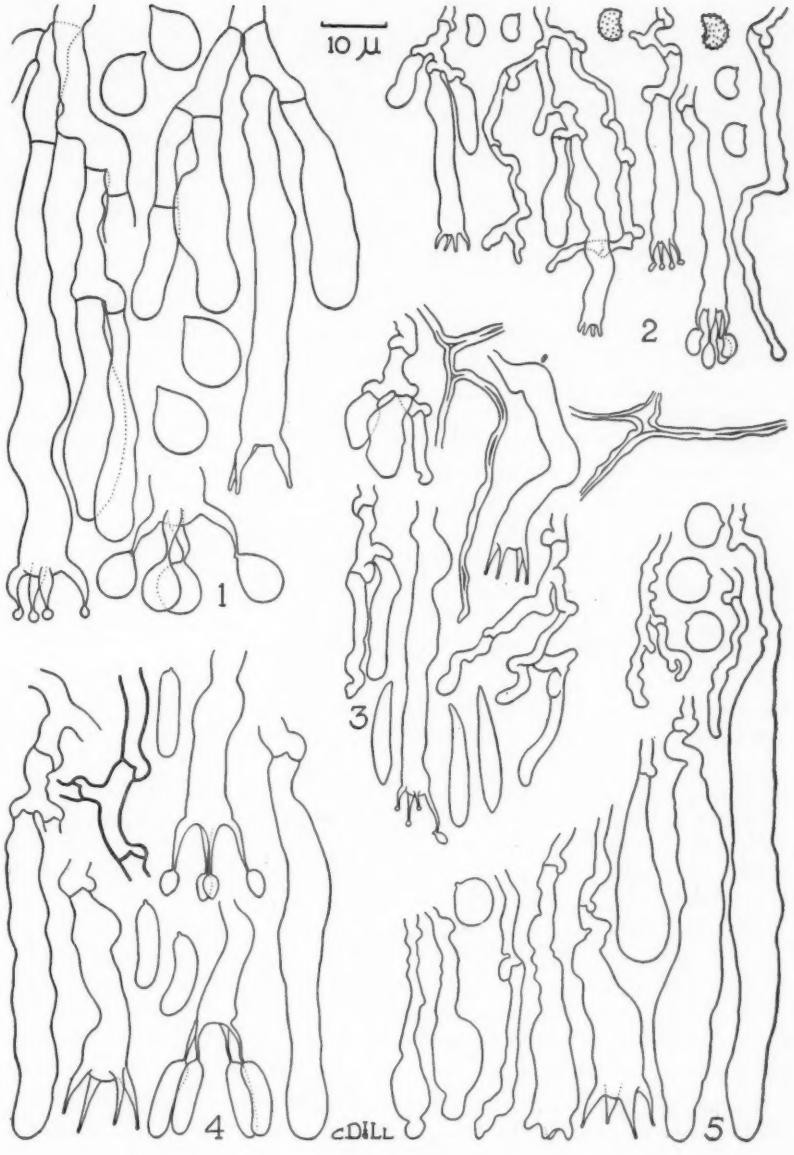
Specimens examined:

Ontario: On decorticated wood of *Pinus* sp. Bear Island, Lake Timagami, Aug. 23, 1939, *14982* **type**; July 21, 1939, *14981*; Aug. 1938, *17698*; Aug. 17, 1943, *19082*, *19085*, *19094*, *19100*; on decorticated wood of *Thuja occidentalis*, Oakland swamp, Brant Co., R.F. Cain, June 12, 1939, *16569*; Oct. 21, 1946, *21394*; Hatchley, Brant Co., Oct. 11, 1943, *18791*; highway between milepost 23 and 24 Algonquin Park, Sept. 17, 1939, *21860*.

This species is apparently quite common on decorticated coniferous wood in Ontario but has not been encountered elsewhere. Branched tortuous hyphae are sometimes included in the hymenium as hyphal-like paraphyses.

FIG. 1. Corticium delectabile, *basidia and spores*. FIG. 2. Corticium electum, *basidia, paraphyses, and spores*. FIG. 3. Corticium praestans, *basidia, spores, and subicular hyphae*. FIG. 4. Corticium definitum, *basidia, spores, gloeocystidia, and basal hyphae*. FIG. 5. Corticium sulfureo-isabellinum *Litsch., basidia, spores, gloeocystidia, and paraphyses*.

All figures were drawn with the aid of a camera lucida at a uniform scale and reproduced at a magnification of approximately 1000×.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Species of *Corticium* having the spore wall strongly amyloid and roughened are not found outside the group of species commonly included by European students of the group in the genus *Gloeocystidium*. It is possible that the species here described may be related to *Gloeocystidium furfuraceum* (Bres.) Höhn & Litsch., which is also common in Ontario. The present species differs in spore size and shape and in the absence of typical gloeocystidia. What appear to be immature basidia often occur having somewhat resinous contents, but these do not develop to structures that would properly be referred to as gloeocystidia.

The spore walls appear definitely roughened when mounted in water or in Melzer's solution but this roughening disappears when potassium hydroxide is included in the mounting medium. A similar reaction has been noted in other cases particularly in the genus *Aleurodiscus*. The now almost universal use of potassium hydroxide – phloxine as a mounting medium by students of this and related groups may result, in such cases, in spore walls being described as smooth unless some other medium is used as a check. The writer has adopted the practice when dealing with an unknown form of always making a crushed mount in Melzer's solution as a test for amyloidity and this procedure answers for observation of obscure roughening of the spore wall as well.

Corticium praestans sp. nov. (Fig. 3)

Fructificatio alba vel cremea, divergens vel e locis orbicularibus coalescentibus emergens, firma, coriacea, tenuis 40–50 μ; subiculum, plerumque e dense intricatis fibrosis hyphis 0.8–2.5 μ diam. crasse tunicatis, luminibus capillaribus aut absentibus, sed etiam partim ex hyphis tenuiter tunicatis, intricatis, nodoso-septatis, tandem basidia gerentibus compositum; gloeocystidia absentia; basidia 25–30 $\times$ 5–6 μ, infra ventricosa vel subventricosa, supra longa cylindracea flexuosa, 4 rectis 5–5.5 μ longis sterigmatibus; basidiosporae subclavatae, subarcuatae, 12–14 $\times$ 2.5–3 μ; tunicis tenuibus, hyalinis, levibus.

Fructification white to cream, spreading, often from coalescing orbicular patches, margin determinate, thin 40–50 μ; in texture firm, felty; subiculum coriaceous, for the most part composed of closely compacted interwoven fibrous hyphae 0.8–2.5 μ in diameter, with thick refractive walls and with lumina capillary or absent, hyaline in water or potassium hydroxide, reddish brown in Melzer's solution, no septa observed, branching irregularly; thin-walled nodulose frequently branched hyphae with clamps scattered in subiculum from which the basidia arise; gloeocystidia absent; basidia at first subglobose, becoming irregularly obpyriform, at maturity ventricose or subventricose below, long cylindrical flexuous above, 25–30 $\times$ 5–6 μ, more or less truncate apically with four straight sterigmata 5–5.5 μ long; basidiospores subclavate, slightly curved, laterally flattened and attenuate toward base, 12–14 $\times$ 2.5–3 μ sometimes adherent in groups of two to four, walls thin, hyaline, smooth, non-amyloid.

Specimens examined:

Ontario: On bark of decaying branches of *Quercus alba* on ground, Petawawa Forest Reserve, Chalk River, Ont., Sept. 12, 1939, *14121* **type**; on stems of *Osmunda Claytoniana*, same locality, Aug. 31, 1941, *17326*; on stems of *Rubus* spp. woods W. of Maple, Oct. 4, 1947, *22080*, *22081*.

Iowa: On oak (?) leaves on ground, Iowa City, Aug. 17, 1943, G. W. Martin *4911*, TRT.

This species belongs in Section 10 Trichostroma of the Bourdot and Galzin classification. They include in this section *Corticium odoratum* (Fr.) B. & G., *C. portentosum* B. & C. and *C. subodoratum* Karst. We would add to this list the species described above, *C. galactinum* Fr. sensu Burt and *C. abeuns* Burt (=*Gloeocystidium ochroleucum* Bres. & Torr.). The latter species had been included by Bourdot and Galzin in a section similarly named in the genus *Gloeocystidium*. All of these species, with the exception of *C. subodoratum*, are characterized by having a coriaceous subiculum made up largely of narrow fibrous hyphae that turn a characteristic reddish brown in Melzer's solution and possess other characteristics that suggest close relationship. *C. subodoratum* Karst. has thick-walled, somewhat fibrous hyphae in the subiculum but these are not comparable to the subicular hyphae in the other species listed and do not give the characteristic reaction in Melzer's solution. For this and other reasons the species is, we believe, quite unrelated. Examination of the type of *C. subodoratum* shows that it is a synonym of *C. rubicundum* Burt.

With this elimination, the species mentioned above all show characters that suggest a relationship with the genus *Vararia* (=*Asterostromella*) and it seems quite probable that ultimately they will be found to form a natural section of that genus or perhaps be united in a separate related genus. Until such time as the species of *Vararia* and their relatives can all be reviewed, it seems best to recognize the section Trichostroma of Bourdot & Galzin and to describe the above species in *Corticium*.

Corticium definitum sp. nov. (Fig. 4)

Fructificatio extensa, tenuis, siccitate minute reticulata, glauco-caerulea dein cinerea; subiculum paulum, hyphae basales 3–4 μ diam., nodoso-septatae, tunicis firmis, partim leviter incrassatis, ad rectam straturam subhymenialem producendum ramosis; cystidia subcylindracea, subflexuosa, 35–50 × 5.5–7 μ immersa vel parum emergentia; basidia subcylindracea, flexuosa, saepe infra ventricosa, 28–40 × 5.5–8.5 μ, 4 sterigmata subulata 6.5–8.5 μ longa gerentia; basidiosporae subcylindraceae vel allantoideae 11–14 × 3–4 μ, tunicis tenuibus hyalinis, levibus.

Fructification extensive, forming a thin interrupted often minutely reticulate growth, blue gray when fresh, smoky when dry, margin indeterminate; basal hyphae few, 3–4 μ in diameter, with clamps, wall thin, firm, or in part slightly thickened, branching at right angles and forming an upright subhymenial layer; gloeocystidia subcylindrical, somewhat flexuous, 35–50 × 5.5–7 μ, arising in the subhymenial layer or directly from basal hyphae, immersed or protruding slightly; basidia subcylindrical, flexuous, frequently ventricose below, 28–40 × 5.5–8.5 μ, at maturity extruded beyond the level of the hymenium of immature basidia, bearing four subulate slightly divergent sterigmata 6.5–8.5 μ long; basidiospores subcylindric to allantoid, 11–14 × 3–4 μ with minute lateral apiculus, walls thin, hyaline, smooth, non-amyloid.

Specimens examined:

Ontario: On decorticated, slightly decayed coniferous wood, woods W. of Maple, York Co., Oct. 18, 1942, *17800* **type**.

The relationship of this species is probably with the section Gloeocystidiales of *Peniophora* in the Bourdot & Galzin classification and to some species of their section Ceracea of *Gloeocystidium*. In this species there is no suggestion of colored resinous secretion characteristic of several of the members of those sections. It matters little, at present, whether forms of this sort are included in *Peniophora* or *Corticium*.

Corticium sulfureo-isabellinum Litschauer, apud Pilát, Acta Musei Nat. Pragae, 2B: 43. 1940. (Fig. 5)

Fructification broadly and irregularly effused, thin 50–90 μ, at first soft ceraceous, subgelatinous, sulphurous or somewhat dingy yellowish, becoming indurated, thinly crustaceous, or submembranous, cream, creamy-alutaceous or isabelline to cinnamon buff, adnate, margin gradually thinning out; hymenium smooth, glabrous, continuous, in age frequently becoming cracked; subiculum somewhat indistinct, basal hyphae horizontal, conglutinate, vertical hyphae densely entangled, with clamps at septa, wall thin becoming collapsed and indistinct; gloeocystidia cylindrical to subclavate 35–75 $\times$ 5.5–8 μ, flexuous; paraphyses in hymenium hyphal-like, flexuous, branching, tortuous to subclavate; basidia broadly clavate, tapering from slender hyphal base, 20–40 $\times$ 7–8.5 μ, bearing four subulate, somewhat arcuate or nearly straight sterigmata 8–10 μ long; spores globose or subglobose, 5.5–7 $\times$ 5–6 μ, slightly compressed laterally with distinct apiculus, hyaline, walls thin, smooth, non-amyloid, multi- or uniguttulate.

Specimens examined:

Ontario: On bark of *Abies balsamea*, Bear Island, Lake Timagami, Aug. 11, 1937, *11950*; R. Biggs, Aug. 26, 1935, *8653*; July 28, 1936, *10467*; portage to Gull Lake, W. mainland, Lake Timagami, R. Biggs, Aug. 6, 1935, *8475*; Aug. 10, 1936, *10466*; Sept. 9, 1936, *10468*; R. F. Cain, Aug. 15, 1946, *20962*; Algonquin Park, Opeongo Lake, R. F. Cain, Aug. 23, 1939, *14246*, *14789*; Smith Lake, Aug. 24, 1939, *14791*; highway near milepost 2, Oct. 13, 1940, *16263*; Sturgeon River, near Beardmore, Thunder Bay District, R. F. Cain, Aug. 29, 1944, *19167*; Petawawa Forest Reserve, Chalk River, Sept. 12, 1939, *14320*; Sept. 5, 1941, *17387*.

Quebec: On bark of *Abies balsamea*, near Camp Mercier, Laurentide Nat. Park, Aug. 27, 1938, TRT.

New Hampshire: On bark of *Abies balsamea*, Bethlehem, July 27, 1936, H. G. Eno, BPI, FP *81330*, TRT.

Austria: On bark of *Abies pectinata*, Tirol, Kranebittenklamm near Innsbruck, V. Litschauer, Aug. 8, 1929, TRT.

Czechoslovakia: On bark of *Abies alba*, Carpatorossia Bily Potok, A. Pilát, Aug. 1935, TRT **type**, comm. V. Litschauer.

When the manuscript for the present contribution was first written, it contained a Latin description of the above species that had been sent to the writer by the late Prof. V. Litschauer of Innsbruck, Austria. At that time we were under the impression that the species had not been published. We are indebted to Dr. D. P. Rogers for sending quite recently a reference to the publication of the species for Litschauer in a paper by Dr. A. Pilát (4), which had not previously come to our notice.

The history of our interest in this species follows: On Jan. 29, 1936 a specimen of a gloeocystidiate *Corticium* collected in the Timagami region in the summer of 1935 (TRT *8475*), which we had been unable to identify, was sent to Prof. Litschauer for his opinion. Referring to this specimen, he replied, Feb. 17, 1936, as follows:

> "Der Pilz, den Sie mir in dem Brief gesendet haben, ist mir schon lange bekannt. Ich habe ihn schon 2 mal in Tirol gesammelt und er ist mir auch von Herrn Dr. Pilát aus der Cechoslovakei zugesendet worden. Es ist der Pilz eine nov. spec., *noch nicht beschriebene Art.* Sie wird demnächst mit anderen Corticiumarten von mir veröffentlicht werden. Es handelt sich nicht um eine Gloeocystidiumart, sondern um ein echtes Corticium. Ich habe die Art *Corticium sulfureo-isabellinum* genannt. Auch Herr Bourdot, den ich die Art schon vor mehreren Jahren zur Begutachtung sandte, hat sie als eine nov. spec. erklärt. Ich sende Ihnen 2 Proben der Art zum Vergleich und lege auch die von mir entworfene Beschreibung der neuen Art bei."

In the description to which he refers above no mention was made of gloeocystidia. This description, which was in Latin, is the same as the one recently published in Pilát's paper. Three years later, in 1939, a portion of the New Hampshire collection listed above was sent Litschauer and his attention was called to the presence of gloeocystidia, which had also been found consistently present in several Ontario collections made in the meantime, as well as in the two European collections which he had sent (listed above). He replied on June 27, 1939 as follows: "Der Pilz der Kollecte ist jedenfalls meine Art. Gloeocystiden kann ich aber keine finden".

In connection with the description published by Pilát, no mention is made of the North American collections that had been sent to Litschauer. It seems probable that Litschauer had sent Pilát a copy of the same descriptive sheet that he had sent to us in 1936 that had evidently been prepared previous to the receipt of the first American specimen.

Careful study has shown that gloeocystidia are consistently present in all collections of this species that we have examined, and mention of them is included in the English description given above. With this major modification together with a few minor changes and some rearrangement our description is essentially a translation of that furnished by him in 1936 and as published by Pilát. Litschauer's failure to note the presence of gloeocystidia may have

been due to the fact that they are not abundant and could easily be overlooked if only sections were examined. They can be readily demonstrated if well prepared crushed mounts are examined in potassium hydroxide–phloxine.

The presence of gloeocystidia together with the character of the basidia and size and shape of spores bring this species very close to *Corticium* (*Gloeocystidium*) *radiosum* Fr. and for a time it seemed doubtful that the two were distinct. Careful comparative study has shown, however, that there are sufficient differences to justify separation. In *C. radiosum* the fructifications are extensive, usually a bright cream yellow and the margin noticeably radiate while in *C. sulfureo-isabellinum* the color is ochraceous or cinnamon buff, and the fruiting body less effused and without radiate margin. In the former the hyphae are typically without clamps and the spores weakly amyloid while in the latter clamps are regularly present and the spores show no evidence of amyloidity. The gloeocystidia of *C. radiosum* are commonly strongly ventricose below, generally slender cylindrical or flexuous above while in *C. sulfureo-isabellinum* they are cylindrical to subclavate, never or rarely ventricose at the base. The former species has a wide host range, occurring commonly on decorticated wood of both conifers and deciduous trees while so far the latter has been collected only on the bark of *Abies* spp.

The drawings for Fig. 5 were all made from the Austrian collection made by Litschauer with the exception of the gloeocystidium second from the right and the two smaller gloeocystidia and the single spore farthest to the left, which were drawn from TRT *16263*.

Corticium eximum sp. nov. (Fig. 6)

Fructificatio in suborbicularibus coalescentibusque areolis, pruinosa vel poroso-reticulata, alba, 50–70 μ crassa; subiculum hyphis 3.5–4.5 μ diam. laxe intricatis, nodoso-septatis; gloeocystidia absentia; basidia 20–25 μ longa, 5.5 μ diam., supra cylindracea, infra ventricosa 7–8.5 μ diam., plerumque 2, raro 3 vel 4 divergentia 6.5–8.5 μ longa sterigmata gerentia; basidiosporae late ellipsoideae 8–10.5 $\times$ 5.5–6 μ, tunicis tenuibus hyalinis levibus.

Fructifications in irregular, orbicular coalescing patches, pruinose to porous-reticulate, and delicately fibrillose at margins, becoming continuous in centers, conforming to the irregularities of the substratum, white, 50–70 μ thick in center; a few hyphae 3.5–4.5 μ in diameter next the substratum are loosely arranged more or less horizontally from which arise upright or ascending branches that branch repeatedly to form a continuous hymenium in the center of the fructifications but more or less interrupted at margin, clamps present at septa, subhymenium made up of shorter and slightly broader cells; gloeocystidia absent; basidia 20–25 μ long, uniformly cylindric in the upper

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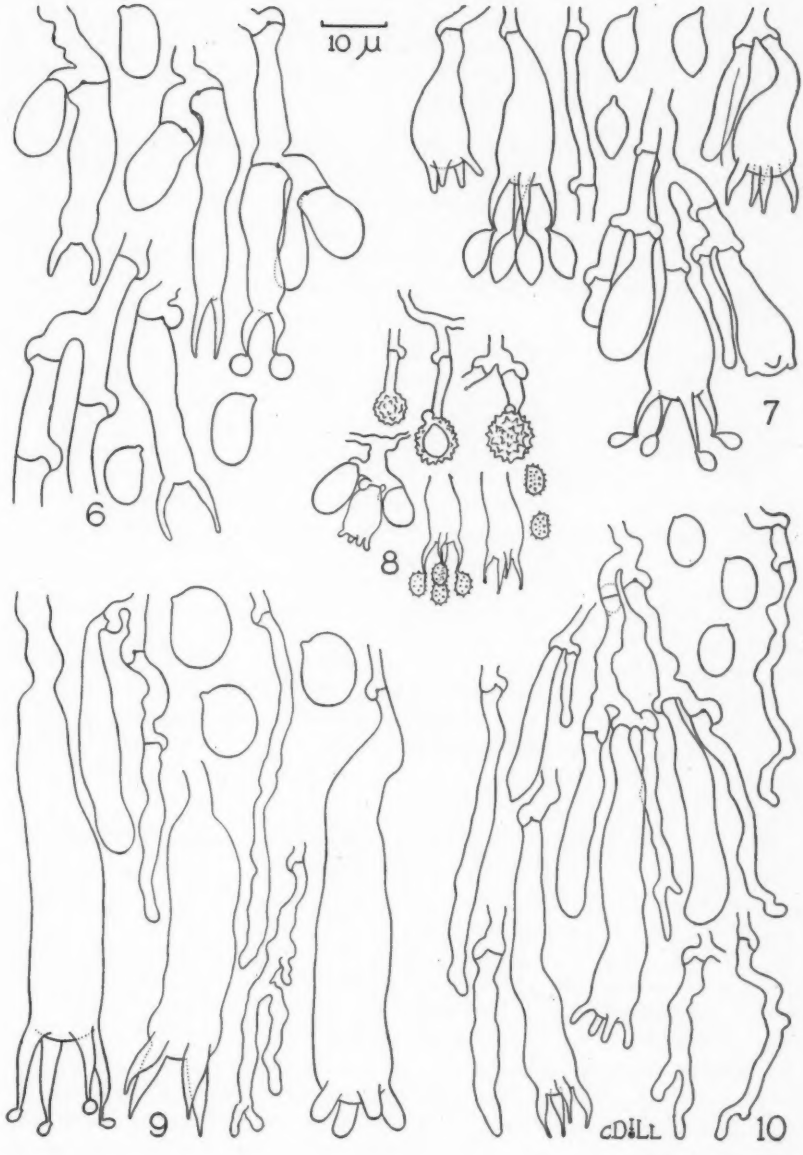
FIG. 6. Corticium eximum, *basidia, spores, and hyphae.* FIG. 7. Corticium reconditum, *basidia and spores.* FIG. 8. Corticium invisitatum, *basidia, spores, and chlamydospores.* FIG. 9. Corticium probatum, *basidia, spores, and paraphyses.* FIG. 10. Corticium notabile, *basidia, spores, and paraphyses.*

All figures were drawn with the aid of a camera lucida at a uniform scale, and reproduced at a magnification of approximately 1000×.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half and 5.5 μ in diameter, ventricose below, 7–8.5 μ in diameter, proliferating from base of clamp, bearing usually only two sterigmata 6.5–8.5 μ long, becoming divergent, rarely three or four; spores broadly ellipsoid, 8–9–10.5 $\times$ 5.5–6 μ, flattened and appearing straight on one side with prominent lateral apiculus, walls thin, hyaline, smooth, non-amyloid.

Specimens examined:

Ontario: On bark of lower dead branches of *Pinus Strobus*, woods near Holland River Marsh, 5 miles W. of Aurora, York Co., May 29, 1937, *12916* **type.**

The relationship of this very distinct species with others of the genus is not at all clear. For the most part the fructifications are associated with old sphaeriaceous fungi. Frequent anastomoses of the basal hyphae were observed and the branches of the basal and upright hyphae often arise from the clamps.

Corticium reconditum sp. nov. (Fig. 7)

Fructificatio tenuis, delicata, isabellina, hypochnoidea, siccitate poroso-reticulata; hyphae subiculi, 1.5–3 μ diam., tenuem parallelem straturam ramis rectis in quibus hymenium consistit producentes, nodoso-septatae; gloeocystidia nulla; basidia late clavata basi antustiora, 16–20 $\times$ 7.5–8.5 μ, tunicis fragili rugosa brunneola resinosa incrustatione tectis, 4 gracilia subulata, late divergentia, 7.5–8 μ longa sterigmata gerentia; basidiosporae lenticulares, lateraliter compressae, 8.5–10 $\times$ 4.5–5.5 μ, tunicis tenuibus hyalinis levibus.

Fructification thin and delicate, isabella color, hypochnoid, becoming minutely porous-reticulate, loosely adherent, margin indeterminate; hyphae of subiculum 1.5–3 μ in diameter, forming a thin horizontal layer, giving rise to erect loosely branched hyphae from which the hymenium is formed, septa with clamps; gloeocystidia none; basidia cylindrical and hyphalike when first formed, becoming swollen above and at maturity broadly clavate with narrowed base 16–20 $\times$ 7.5–8.5 μ, wall frequently covered by delicate rugose brownish resinous incrustation that gives the color to the fructification, bearing four spores on slender subulate widely divergent sterigmata, 7.5–8 μ long; basidiospores lenticular, laterally compressed on one side with prominent apiculus 8.5–10 $\times$ 4.5–5.5 μ, walls thin, hyaline, smooth, non-amyloid.

Specimens examined:

Ontario: On bark of *Pinus Strobus*, Petawawa Forest Reserve, Chalk River, Renfrew Co., Sept. 12, 1939, *17585*, **type.**

The relationship of *C. reconditum* to others in the genus is not clear. Superficially the fructifications resemble those of *Coniophora*. The spores however are entirely colorless and not of the type characteristic of that genus. The color is in part due to a delicate incrustation of brown resinous material, soluble in potassium hydroxide, mostly occurring on the walls of the basidia, that is left after the collapse of old basidia as an obscure brown granular disorganized mass within the hymenium.

The spores are characteristic, sublenticular to subfusiform almost biapiculate, and suggestive of the spores of some strains of *Pellicularia vaga* (Berk. & Curt.) Rogers. The species, however, has no relation to those included in the genus *Pellicularia*.

Corticium invisitatum sp. nov. (Fig. 8)

Fructificatio effusa, tenuis, alba, pruinosa vel submembranacea; subiculum paulum, hyphis basalibus paucis, paralleliter currentibus, rectos ramos qui in apice racemus basidiorum vel chlamydosporarum fit ferentibus, hyphis 2–3 μ latis, nodoso-septatis, tunicis tenuibus ampulliformibus interdum tumosis; chlamydosporae numerosae, 5.5–7 μ, in apice ramorum hypharum productae, globosae vel subglobosae, tunicis incrassatis 0.8–1.5 μ, rugosis, hyalinis; basidia oblonga vel cylindracea vel subclavata 8.5–12 × 4.5 μ, 4 gracilibus 3.5–4.5 μ longis sterigmatibus; basidiosporae late ellipsoideae vel subreniformes, lateraliter compressae, 3.5–4.5 × 2–3 μ, tunicis hyalinis asperulatisque.

Fructification thin, white, pruinose to submembranous, occurring in broadly effused areas over substratum, loosely adherent, friable, margin indeterminate; subiculum scanty, basal hyphae few, horizontal, giving rise to lateral perpendicular branches that terminate in clusters of basidia or chlamydosporophores; subhymenium with considerable imbedded crystalline material, hyphae 2–3 μ broad, walls thin, septa with clamps, ampulliform swellings occasionally present; chlamydospores numerous, 5.5–7 μ in diameter, developing terminally on hyphal branches generally subtended by clamp, globose or subglobose, wall thickened 0.8–1.5 μ, rugose, hyaline; basidia oblong to cylindrical or subclavate 8.5–12 × 4.5 μ bearing four slender straight or slightly arcuate sterigmata 3.5–4.5 μ long; basidiospores broadly ellipsoid to subreniform, laterally compressed, broadest at distal end, with minute apiculus, 3.5–4.5 × 2–3 μ, wall hyaline, asperulate, non-amyloid.

Specimens examined:

Ontario: On duff and debris on ground in old shack, S.E. of Hatchley, Brant Co., Sept. 3, 1943, R. F. Cain, *18792* **type.**

A member of Bourdot & Galzin's Section 11 Humicola of *Corticium*, this species is unique because of the presence of thick-walled spores, termed chlamydospores in the above description, which apparently readily become detached and function as conidia. The nature of the sculpturing on the outer wall of these spores is difficult to determine but there is some evidence that the rugose markings may be in the nature of spirally arranged ridges not well indicated in the drawings of Fig. 8.

The genus *Phlebiella* Karst., as noted in the introduction, is available for this group of species. Until the group as a whole can be adequately reviewed it seems best to continue to recognize the section Humicola and to describe new forms in *Corticium*.

Corticium probatum sp. nov. (Fig. 9)

Fructificatio effusa, cremea vel eburneo-citrula, tenuis, 80 μ, siccitate membranacea; subiculum, hyphis 2–3 μ diam. laxe intricatis, nodoso-septatis; gloeocystidia nulla; paraphyses velut ramosae flexuosaeque hyphae praesentes; basidia magna, 40–65 × 10–12 μ, subcylindracea vel late clavata, subflexuosa, 4-subulatis subarcuatis 9–12 μ longis sterigmatibus; basidiosporae ovoideae vel subglobosae, 9–12 × 8.5–10 μ, apiculo laterali prominente lateraliter depressae, tunicis hyalinis tenuibus levibus.

Fructification broadly effused, cream to ivory yellow, thin, 80 μ, smooth, soft membranous when dry, somewhat separable, margin determinate, not differentiated or scarcely so as a narrow delicately fibrillose white border; subiculum of loosely interwoven hyphae 2–3 μ in diameter, septa with clamps, rosettes of crystals imbedded in subiculum; gloeocystidia none; simple or

branching flexuous hyphae extending into hymenium as paraphyses; basidia large, 40–65 × 10–12 μ, subcylindric to broadly clavate, slightly flexuous, narrowed at base, extruded beyond level of hymenium when mature, contents conspicuously guttulate, bearing four subulate slightly arcuate sterigmata 9–12 μ long; basidiospores ovoid to subglobose, 9–12 × 8.5–10 μ, depressed on one side with prominent lateral peglike apiculus, walls colorless, thin, smooth, non-amyloid.

Specimens examined:

Ontario: On decayed coniferous wood, woods W. of Maple, York Co., Oct. 21, 1939, *17589* **type**.

C. probatum is another species with large *Aleurodiscus*-like basidia, the relationship of which with other species is not clear. The large basidia, hyphalike paraphyses and the characteristic, rather large spores, form a combination of characters that serve to separate it from other described species.

Corticium notabile sp. nov. (Fig. 10)

Fructificatio albida vel eburneo-citrula, subceracea, siccitate subindurata, in panniculis elongatis producta, dein rimosa, adnata, usque ad 650 μ crassa, margine determinato; subiculum obscure stratosum, hyphis fere rectis, 1.5–3.5 μ diam., nodoso-septatis, tenuiter tunicatis compositum; basidia clavata 40–50 × 7–8 μ, quattuor subulata sterigmata 7.5–8.5 μ gerentia; basidia cum paraphysibus velut hyphis, interdum subacuminata, saepe nodulosa, interdum furcata, vel obtusis furcis; basidiosporae ovoideae 7–9 × 4.5–6 μ, tunicis tenuibus hyalinis levibus.

Fructification whitish to ivory yellow, subceraceous, somewhat indurated when dry, occurring in elongate irregular patches, to 650 μ thick, becoming rimose, adherent; margin determinate and undifferentiated; subiculum not sharply differentiated into layers, basal horizontal portion of hyphae with thickened, highly refractive, somewhat gelatinized walls from which arises an indistinctly stratose portion of vertical hyphae that ultimately form the hymenium; upright hyphae 1.5–3.0 μ, thin-walled, with clamps, interwoven with granular-appearing collapsed hyphae; basidia clavate, somewhat flexuous 40–50 × 7–8 μ, bearing four subulate nearly straight sterigmata, 7.5–8.5 μ long, accompanied by hyphalike paraphyses sometimes subacuminate, often nodulose, occasionally furcate, or with stout prongs; basidiospores ovoid, 7–9 × 4.5–7 μ slightly compressed laterally with minute apiculus, walls thin, hyaline, smooth, non-amyloid.

Specimens examined:

Ontario: On decorticated wood of *Picea mariana*, Ko-ko-ko Bay, Lake Timagami, July 19, 1938, *13682*, *13689*; R. F. Cain *13687*; Aug. 3, 1939, *15005*, *14911*, **type**; July 21, 1943, *18668*; Petawawa Forest Reserve, Chalk River, Sept. 2, 1941, *18060*; on *Pinus Banksiana*, Constance Bay, Ottawa River, June 30, 1939, *15167*; Sturgeon River, near Beardmore, Thunder Bay District, R. F. Cain, Aug. 30, 1944, *19169*; on *Thuja occidentalis*, woods S. of Aurora, York Co., Oct. 1, 1939, *16548*; near Friday's lodge W. shore of Lake Timagami, R. F. Cain, Aug. 12, 1939, *14969*; on coniferous wood, Bear Island, Lake Timagami, R. Biggs, Aug. 10, 1935, *16754*; Aug. 16, 1935, *8215*; N. of Mt. Albert, York Co., Oct. 28, 1938, *13688*.

Nova Scotia: On deciduous wood, U. Brookside, Colch Co., July 4, 1935, L. E. Wehmeyer *385*, TRT.

Manitoba: On *Pinus Banksiana*, Victoria Beach, I. Mounce & G. R. Bisby, June 22, 1935, OTB F*6324*, TRT; Sandilands Forest Reserve, G. R. Bisby, Oct. 4, 1936, OTB F*7056*, TRT; on *Picea*, 13 miles E. of Beausejour, G. R. Bisby and A. H. R. Buller, Sept. 29, 1935, OTB F*6431*, TRT.

Connecticut: On *Castanea dentata*, N. Bloomfield, H. G. Eno, June 18, 1937, BPI, FP *82347*, TRT; Black Rock State Forest, Watertown, H. G. Eno, Aug. 23, 1939, BPI FP*84825*, TRT.

California: On coniferous wood, near summit of old LaHonda Rd., San Mateo Co., Nov. 13, 1942, M. Doty *3388*, TRT.

This rather conspicuous species has proven so common that it seems surprising that it has not been described previously. All of the collections recorded from Ontario were made on coniferous wood. We have included three collections from Nova Scotia and Connecticut occurring on wood of deciduous trees that seem to be the same. The gross characters of the fructifications together with the presence of abundant hyphal paraphyses and other microscopic details serve to distinguish this species from others with which we are familiar.

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FASCIATION OF SWEET PEAS[1]

By E. H. Garrard[2]

Abstract

A description is given of fasciation in a sweet pea plant, from which a strain of *Agrobacterium tumefaciens* (Smith and Townsend) Conn was isolated. Germinated and nongerminated sweet pea seed inoculated with the organism were grown in agar, sand, and soil. Fasciation, dwarfing, swollen roots and stems as well as yellowed and crinkled leaves were produced in most of the plants grown in agar and sand. Fewer plants grown in soil were affected. Microscopical examinations revealed organisms in fasciated areas, roots, and stems. The organisms were recovered in each case by plating with Congo red agar.

Fasciation was not produced in garden pea plants although some plants were dwarfed. Typical galls were not produced on stems of sweet and garden pea plants by needle inoculations. A description of the organism is given and certain features of its action on sweet pea plants are discussed.

Introduction

Fasciation of sweet peas has been reported on various occasions and different organisms have been claimed to be the cause of the disease. Brown (1) described fasciation of sweet pea plants from New Jersey and New York States and of garden pea plants from Maryland and Virginia caused by a weak or highly specialized strain of *Agrobacterium tumefaciens*. Sweet pea fasciation is reported by Muncie and Patel (4) to be caused by *Agrobacterium tumefaciens*, certain fungi, and other environmental factors. Tilford (7) isolated *Xanthomonas fasciens* as the cause of fasciation and failed to produce typical fasciation in sweet peas by inoculation with the crown gall organism. Lacey (2) isolated an organism similar to *Xanthomonas fasciens* associated with fasciation of sweet pea and other plants.

This paper deals with fasciation of sweet peas caused by an organism resembling *Agrobacterium tumefaciens* and presents certain features to which little reference has been made in the literature.

Description of Original Specimen

The original sweet pea plant submitted for examination was 10 in. in height; the stem was spindly and the leaves were dwarfed, yellow, and somewhat shrivelled. A leafy gall-like structure, composed of distorted leaves and shoots, was situated at the crown of the plant. It was compressed into an irregularly shaped mass approximately 2.5 cm. in diameter. The interior was light green in color and quite firm.

Enquiries revealed that 40 plants, representing one-third of the crop, showed similar symptoms of dwarfing, with spindly stems and general lack

[1] *Manuscript received December 22, 1947.*
Contribution from the Department of Bacteriology, Ontario Agricultural College, Guelph, Ont.

[2] *Professor and Head Department of Bacteriology*

of vigor and color. The sweet pea plants were grown from imported seed and consisted of a mixture of 12 early or winter flowering varieties. They were sown in loam composed of rotted sod and later transferred to a permanent bed in the greenhouse, the soil in which was composed of a mixture of muck, rotted sod, and well rotted manure. No other legumes previously had been grown in the same soil.

Isolation of Causal Organism

Microscopical examination of various portions of the abnormal tissue revealed the presence of numerous, medium-sized, Gram-negative rods of various shapes. These included curved forms, rods with swollen ends, *y* forms, and other bacteroidal shapes similar to those described by Smith (6). Sections of the tissue were macerated in sterile water and plated with Congo red agar. After seven days' incubation at 25° C. (77° F.) numerous dark red colonies appeared on all the plates. Representative colonies were transferred to slants for further observation. Microscopical examinations of the cultures showed the same rod-shaped organism without involution forms; star-shaped grouping, however, was quite common.

Artificial Inoculations

A. Agar Cultures

Mixed, winter flowering sweet pea seeds were sterilized by submersing them in alcohol for one minute, followed by mercuric chloride 1 : 1000 for seven minutes under vacuum, with several changes of sterile water. The seeds were germinated on moist blotting paper in sterile Petri dishes. They then were transferred with flamed forceps to 2 in. diameter test tubes containing modified Crone's agar. A drop of suspension of the isolated organism was deposited on each seed. The test tubes were wrapped in brown paper and maintained for a period of approximately six weeks. Modified Crone's solution was added from time to time.

Twenty-six of 32 plants developed fasciation or other signs of abnormality. First signs of fasciation appeared about the end of the third week and from that time on, the plants exhibited various effects of the action of the organism. These included dwarfing, thin or swollen roots, lack of roots, and crinkled, yellowed leaves. These are illustrated in Plate I. Fig. 1 shows the development of fasciation on a plant in a test tube at approximately four weeks. Fig. 2 shows the tallest of the fasciated plants (10 in.) with an uninoculated control plant. Fig. 3 presents an enlarged view of the fasciated area of the plant shown in Fig. 2. In Fig. 4 is shown a six-weeks-old plant that developed a poor root system and no top. Fig. 6 shows an enlarged view of the top of the plant shown in Fig. 2 where the leaves are crinkled and the stem is swollen at one point.

Microscopical examinations of swollen portion of the stem, fasciated area at the crown, and of various sections of the roots revealed characteristic

bacteria in great numbers. No difficulty was experienced in recovering them from all three locations on Congo red agar.

B. Sand Cultures

The experiment was repeated with sweet pea seed grown in sand. Washed quartz sand was sterilized in shallow trays at 350° F. for 10 hr. and placed in wide mouthed 3 in. glass jars. The seeds were sterilized as before and germinated, as well as nongerminated, seeds were deposited 1 in. below the surface. They were inoculated by adding a drop of suspension of the organism on the seed before covering over. Controls were included as well as seeds inoculated with a true strain of *Agrobacterium tumefaciens* procured from Dr. M. P. Starr, Hopkins Marine Station of Stanford University. The jars were wrapped in brown paper and held for six to eight weeks. Modified Crone's solution was added daily.

All but one of 18 inoculated seeds developed plants with fasciation or other signs of abnormality. Several seedlings attained a height of 1 in. and died. Examination of such seedlings revealed countless numbers of organisms in all parts of the shoots. A few plants developed fairly normal tops, but possessed two or more dwarfed or swollen shoots arising from a gall-like area at the crown. Others failed to attain a height of more than 2 to 4 in. and exhibited flattened or swollen shoots with few or no roots. Examples of this latter type are shown in Fig. 7.

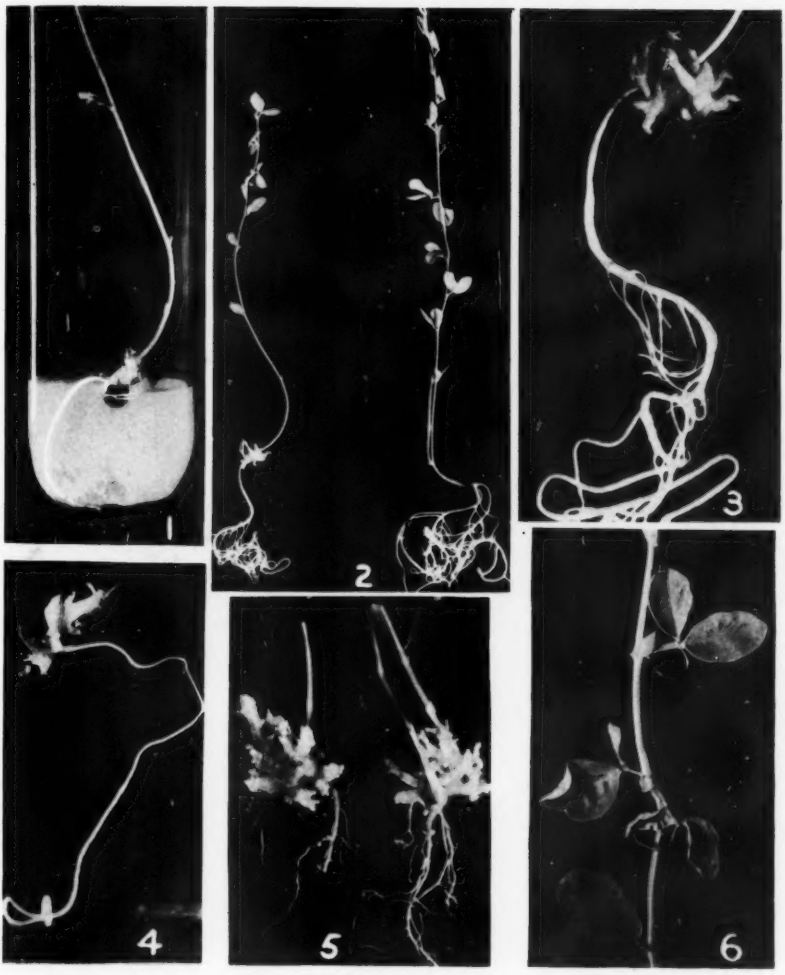
Fig. 5 shows the fasciated areas of two plants that contained nodular swellings on the roots. Although dark in color, they somewhat resembled *Rhizobium* nodules but contained the same organisms found in fasciated shoots, roots, and stems of other plants. Moreover, organisms isolated from these swellings produced typical fasciation in other seedlings inoculated at a later date.

No sign of fasciation or abnormality was found in the seedlings inoculated with the true strain of *Agrobacterium tumefaciens* and the plants compared favorably with the controls, which attained an average height of 18 to 20 in. No difference could be found between germinated and nongerminated seed as plants from both groups developed typical symptoms.

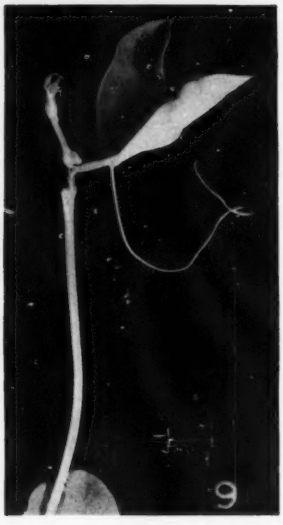
C. Soil Cultures

Sterilized germinated and nongerminated seeds were sown in 4 in. pots containing sterilized garden soil. More than 150 seeds were used, four to each pot. Seeds were inoculated with the isolated organism and others with the true strain of the crown gall organism. They were held for eight weeks.

The results were not so conclusive as in the preceding experiments. Approximately 50% of the plants were affected and dwarfing and yellowing of the plants were more common than gall-like areas at the crown. Certain plants died when they were 2 to 3 in. high and others never attained a height of more than 5 in. Fig. 8 shows three of these plants at six weeks of age. The control plants attained an average height of 14 to 16 in. The plants inoculated



FIGS. 1 to 6. *Fasciation of sweet peas from inoculated seed. 1. Fasciation of four-weeks-old plant in test tube. 2. Six-weeks-old fasciated plant and control, grown on agar. 3. Enlarged view of fasciated area of plant shown in Fig. 2. 4. Six-weeks-old plant without top, grown on agar. 5. Fasciated area of plants grown in sand. 6. Enlarged view of top of plant shown in Fig. 2.*



FIGS. 7 to 9. *Fasciation of sweet pea plants from inoculated seed. 7. Dwarfed fasciated plants grown in sand. 8. Dwarfed forms grown in soil. 9. Garden pea stem showing swelling as a result of needle inoculation.*

with *Agrobacterium tumefaciens* showed no signs of the disease and were as healthy and vigorous as the controls.

Microscopical examinations of the stems of the plants shown in Fig. 8 revealed typical organisms that were recovered on plating.

Examination of Soil

Although it was not possible to obtain any of the original sweet pea seed, a representative sample of soil in which the original plants were grown was obtained. Attempts to isolate the organism directly from the soil resulted in failure. Sterilized germinated seed grown in the soil resulted in only one fasciated and dwarfed plant being obtained, from which typical organisms were recovered.

Garden Pea Inoculations

Garden pea seeds were sterilized, germinated, inoculated, and sown in sterile sand. They were held until the controls formed pods. No cases of fasciation developed, but a few of the plants never attained a height of more than 4 in. The roots on these plants were swollen, the stems were spindly, and the leaves slightly yellowed. Organisms were recovered from the roots and lower parts of the stems.

Gall Production

In an attempt to determine whether the organism was capable of producing typical galls on the upper parts of the plant, both sweet and garden pea control plants were inoculated by needle in various portions of the main stem and petioles. Inoculations also were conducted with the true crown gall organism. While the latter organism produced definite galls about the size of a pea, only one somewhat inconclusive swelling was produced by the isolated organism on the upper part of a garden pea plant. This is shown in Fig. 9 and it somewhat resembles the swelling found on the upper part of the stem shown in Fig. 6, which resulted from seed inoculation. Organisms were recovered from this swelling on Congo red agar.

Description of the Organism

Repeated biochemical and cultural tests with the organism have revealed that it differs only slightly from most descriptions of *Agrobacterium tumefaciens*. Therefore it would appear that the organism is similar to that found by Brown (1), although she does not describe the organism in detail, and that it is a weak or highly specialized form of the crown gall organism. The following is a description of the organism.

A medium sized rod, measuring $0.5 - 0.8 \times 1.5 - 3.0\ \mu$, motile by one to two polar flagella; no spores; capsules; Gram-negative; grouped singly and in pairs; bacteroidal forms found in carbohydrate media. Aerobic; nutrient agar colonies—grayish-white, circular, raised, glistening, entire, becoming viscid; Congo red agar—raised, circular, glistening, and strongly

absorbs the dye; nutrient broth—moderately turbid with suspended filaments, fragile pellicle; gelatin—not liquefied; nitrates—not reduced; indol—not produced; H_2S—not produced. Growth in Uschinsky's medium, no growth in Cohn's solution. No gas in carbohydrate media but slight acid in arabinose, galactose, mannitol, and mannose. B.C.P. milk—neutral to alkaline, no coagulation but definite serum zone; optimum temperature—25° to 30° C.

Discussion

The symptoms of fasciation and prolepsis of sweet pea plants here described generally resemble descriptions of the disease by other workers. Certain differences have been observed, however, that are of particular interest. In the first place Smith (5) refers to the almost complete sterility of fasciated shoots in plants, and Brown (1) reports that microscopical examination of fasciated shoots failed to reveal the presence of microorganisms, although they were isolated on plating. Also, it has been generally observed that primary inoculations with gall-forming strains into certain hosts may result in secondary galls in which no typical organisms are found.

In this series of experiments there has not been any difficulty in demonstrating countless numbers of characteristic organisms in fasciated shoots, stems, or roots and subsequent agar plates in high dilutions have been crowded with colonies While in some plants, more sturdy than others, the organisms were localized in the fasciated areas, most of the stunted and dwarfed plants revealed organisms in the extreme tips of the shoots and in the swollen root ends. The fact that many distorted seedlings with organisms distributed throughout the tissues died quite rapidly demonstrates the unusual invasive activity of this particular strain. Also, such activity no doubt accounts for the pronounced dwarfing and sickly appearance of more mature plants.

The majority of cases of fasciation in plants by artificial inoculation have been effected by needle inoculation (1, 3, 5). In sweet peas, Brown (1) allowed seeds to germinate and then made needle inoculations into the tender shoots. With the exception of needle inoculations into stems to try to produce galls, all inoculations in these experiments were made by adding a suspension of the organism directly to germinated or nongerminated seeds.

Among numerous descriptions of fasciation in plants there is little or no mention of swellings on roots or stems. Swollen roots and nodular protuberances, as shown in Figs. 3 and 5, were quite common in these experiments. Swollen areas on the stems, as shown in Fig. 6, were present but not so commonly encountered. Examination of each swelling always revealed characteristic organisms.

It is not known how common sweet pea fasciation is in Ontario, nor could it be determined whether the trouble was seed- or soil-borne. The above findings, however, do corroborate the fact that organisms other than *Xanthomonas fasciens* are capable of causing fasciation and abnormal development in sweet pea plants.

Acknowledgments

The author wishes to express his gratitude to Professor F. Chase and Mr. F. Newbould, Department of Bacteriology, Ontario Agricultural College, for their assistance.

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STUDIES ON THE METABOLISM OF *STREPTOMYCES GRISEUS* IN RELATION TO THE PRODUCTION OF STREPTOMYCIN[1]

By H. M. Eiser[2] and W. D. McFarlane[3]

Abstract

A synthetic medium inducing high streptomycin production was evolved by studying growth factor and nitrogen requirements of the mold *Streptomyces griseus*. It was concluded that mycelium growth and streptomycin production do not necessarily parallel each other. Histidine appeared to affect both streptomycin and mycelium formation and was essential in any amino acid combination to induce either a high mycelium or streptomycin yield. Valine was shown to stimulate streptomycin synthesis and aspartic or glutamic promoted only mycelium production. Experiments were done to show which metabolic changes in the medium could be associated with growth and which with the antibiotic production.

The effect on the mold of high concentration of sodium chloride was investigated. It was found that by reducing the amount of salt in the nutrient media, the greater part of the streptomycin could be recovered from the mycelium instead of the medium. It appears that the antibiotic is a product of intracellular synthesis, since ions of the lyotropic series affecting the permeability of the cellular membrane affect the distribution of the antibiotic between medium and mycelium.

Introduction

It has now been definitely established that a considerable number of the actinomycetes isolated from soils or other natural substances have the capacity to inhibit the growth of microorganisms. Streptomycin (8) is the most important antibiotic so far obtained from actinomycetes. Its antibacterial spectrum includes both Gram-positive and Gram-negative organisms; moreover in pure form its toxicity is low. With a view of increasing the yield of the antibiotic, research was directed along these main lines:

(*a*). To isolate more active strains, giving higher antibiotic titers.

(*b*). To study the specific nutrient requirements for the synthesis of streptomycin.

(*c*). To develop better methods of isolating the antibiotic from the medium.

The two latter lines of investigation went hand in hand, because the complexity of the media as regards organic constituents made the isolation of streptomycin difficult; the recovery was poor and the isolated product was colored and often toxic at the dosage used for clinical treatment.

The medium first used for the production of the antibiotic was that of Waksman and co-workers (5). Other media (7, 3, 9) have been developed, but none of these has been a defined, synthetic medium. Neither the mechanism

[1] *Manuscript received November 6, 1947.*

Contribution from the Department of Chemistry, Macdonald College, Macdonald College P.O., Que.

[2] *Formerly research assistant, Macdonald College; at present, chemist, Development Department, Distillers Corporation Seagram, Ville LaSalle, Que.*

[3] *Formerly Professor and Head of the Department of Chemistry, Macdonald College; at present, Research Director, Victory Mills, Toronto, Ont.*

involved in the production of the antibiotic, nor the nutrients required for its synthesis are as yet known but it appears that some of the active constituents of meat extract (Waksman's medium) are inorganic (7).

This study was undertaken to develop new media, preferably of known composition, to study the metabolism of the organism when grown in these media, to elucidate the mechanism of streptomycin production, and finally to investigate the observation that sodium chloride has an effect on the distribution of streptomycin between medium and mycelium.

Procedure

The strain *Actinomyces griseus 10* (Waksman)* was used throughout the investigation. The organism was grown in test tube soil culture and slants (nutrient agar) for experimental work were inoculated from the soil culture. Submerged culture was used throughout by agitating test tubes (25 × 200 mm.) in a slanting position. This method offered several advantages, e.g., the small volume gave greater economy, especially when working with amino acids, easier handling, a more uniform inoculum (one inoculation slant served 40 test tubes.) The tubes each containing 10 ml. of medium were autoclaved at 15 lb. for 15 min. They were inoculated with 0.2 ml. of spore suspension (10 ml. of sterile water per test tube slant, spore suspension) and placed at a slant of 23 degrees horizontal. This angle proved best, giving a yield of streptomycin equivalent to that obtained with 50 ml. of medium in 250 ml. Erlenmeyer flasks on the same shaker and within the same period of time. The shaker had a backward and forward motion (84 strokes per minute) and was in a constant temperature (26.6° C.), constant humidity room (75% relative humidity). When streptomycin production reached a maximum (assay), the tubes were removed from the shaker, and the original volume of the medium was restored to compensate for loss due to evaporation. The mycelium was separated by centrifuging, the medium placed in the refrigerator, and samples withdrawn for analysis.

To estimate the streptomycin potency of the medium, a technique based upon the assay of penicillin by Foster and Woodruff (1) was employed, taking into consideration the special precautions observed in the assay of streptomycin as outlined by Loo and co-workers (4).

Glucose was determined by Hanes's (2) modification of the Hagedorn Jensen method. Hydrogen ion concentration was measured with a Hellige pH meter. The media were all adjusted to pH 6.8 to 7.0 before each run. Histidine was determined according to Macpherson's (5) modification of the Hanke–Koessler reaction. Ammonia was measured by the method of Van Slyke and Cullen (11) using 20% sodium carbonate and aerating for at least four hours and usually overnight to ensure complete distillation. Amino nitrogen was determined by the method of Van Slyke as outlined in the

* *Kindly supplied by Dr. G. A. Grant, Ayerst, McKenna and Harrison Ltd., Montreal, Que.*

A.O.A.C. Book of Methods (10). The weight of mycelium was determined by filtering or centrifuging, washing thoroughly and weighing after drying at 80° C.

Results

Nitrogen Requirements

Before studying in detail the nitrogen requirements of *Streptomyces griseus*, it was thought advisable to examine the growth factors as to their effect on streptomycin production. The basal medium was made up as follows:

Glycine	0.4% (equal in nitrogen content to 0.5% peptone)
Glucose	1.0%
Potassium nitrate	0.05%
Sodium chloride	0.5%
Sodium dibasic phosphate × 12 H_2O	0.18%
Magnesium chloride × 6 H_2O	0.05%
Iron chloride × 6 H_2O	0.003%
Copper sulphate	0.001%

The growth factors were added individually and streptomycin and mycelium production were measured. Inositol increased the yield of both streptomycin and mycelium, riboflavin and nicotinic acid seemed to have some effect on stimulating streptomycin production but none on mycelium formation. Folic acid, pyridoxine, pantothenic acid, thiamine, and *p*-aminobenzoic acid showed no stimulatory effect whatsoever. The results, even with inositol, were significantly lower than those obtained with Waksman's medium, thus indicating the need for a detailed study of the nitrogen source.

As pointed out by Waksman (12) actinomycetes develop most readily if complex nitrogenous substances such as proteins are included in the medium. All naturally occurring amino acids were incorporated individually into the basal medium mentioned, in amounts so that the total nitrogen content was equal to that of a 0.5% solution of peptone. An exception was made in the case of histidine and tryptophane since the utilization of the ring nitrogen was in doubt. The efficiency of the individual acids in inducing mycelium and streptomycin production was not confined to any particular group. Histidine gave the best antibiotic production. The optimum ratio of histidine to glucose was next studied. Mycelium production was estimated visually, a value of 5 representing the optimum growth for that experiment (Table I).

To obtain still higher yields of the antibiotic, combinations of amino acids were tested in which each of the amino acids contributed one-third of the total nitrogen equivalent to that in a medium containing 0.5% peptone (Table II).

All combinations containing valine proved superior. The histidine–valine–arginine medium was studied further by varying the concentration of each. If the amount of histidine was kept constant and the valine concentration varied, there was a direct correlation between valine concentration and streptomycin production but not with mycelium formation. The concentration

of histidine must be above a certain level if this is to be observed. If the amount of valine was constant and the amount of histidine varied, there was a direct correlation between histidine concentration and streptomycin and mycelium production. Arginine seemed to have little effect on the production of streptomycin or mycelium.

TABLE I

THE EFFECT OF THE CARBON–NITROGEN RATIO (GLUCOSE HISTIDINE) ON THE PRODUCTION OF STREPTOMYCIN AND MYCELIUM

Medium*		Zone of inhibition, mm.	Mycelium production
Histidine monohydrochloride, %	Glucose, %		
0.1	1.0	19.0	1
0.2	1.0	20.5	3
0.5	1.0	23.0	4
1.0	1.0	24.5	5
2.5	1.0	22.0	4
0.7	1.5	24.5	5
0.7	1.3	25.5	5
0.7	1.0	23.5	5
0.7	0.7	22.0	4

* *In addition the medium contained inorganic salts and 0.0005% inositol.*

TABLE II

THE EFFECT OF COMBINATIONS OF AMINO ACIDS ON STREPTOMYCIN PRODUCTION

Addition to basal medium*	Zone of inhibition, mm.
0.23% histidine, 0.23% valine, 0.22% proline	24.0
0.23% histidine, 0.1% arginine, 0.33% tyrosine	20.0
0.23% histidine, 0.23% threonine, 0.18% lysine	22.0
0.23% histidine, 0.1% arginine, 0.23% valine	26.0
0.23% histidine, 0.23% proline, 0.33% tyrosine	20.5
0.23% histidine, 0.23% valine, 0.23% threonine	25.5
0.23% histidine, 0.23% proline, 0.18% lysine	23.0

* *Basal medium: 1.3% glucose, inorganic salts, inositol.*

The medium containing 0.23% histidine, 0.23% valine, and 0.1% arginine was compared with Waksman's medium and the zone of inhibition expressed in 'S' units of streptomycin.* Waksman's medium produced 180 'S' units, whereas the synthetic medium gave up to 250 'S' units per ml. of medium.

* *An 'S' unit of streptomycin is the amount of the antibiotic that will inhibit the growth of Escherichia coli in 1 ml. of nutrient broth.*

In this investigation it was observed that replacing valine with aspartic acid greatly enhanced the mycelium production but lowered significantly the yield of the antibiotic. When the metabolism of *Streptomyces griseus* was studied, using a complex medium as substrate, it was found (13) that maximum streptomycin production coincided with maximum mycelium formation. It was hoped that a detailed study of two synthetic media, one supporting the streptomycin production and the other mycelium production, would show which metabolic changes were associated with growth and which with the formation of the antibiotic.

The two media had the following composition:

Medium I	Medium II
0.23% Valine	0.23% Aspartic acid
0.23% Histidine	0.23% Histidine
0.10% Arginine	0.10% Arginine
1.30% Glucose	1.30% Glucose
Inorganic salts	Inorganic salts
0.0005% Inositol	0.0005% Inositol

The results are presented in tabular form, the percentage is expressed relative to the highest value obtained for any one variable with both media, which is rated as 100% (Tables III and IV).

TABLE III

METABOLIC CHANGES DURING GROWTH IN A HISTIDINE–VALINE–ARGININE MEDIUM

Analysis of medium	Before sterilization	After sterilization	Duration of fermentation, days					
			1	2	3	4	5	7
pH	6.90	6.90	6.90	6.80	6.75	6.70	6.85	8.50
Residual glucose (mgm. per ml.)	12.2	12.4	12.4	11.3	10.6	8.8	6.25	0.60
Mycelium production (mgm. per 20 ml.)	—	—	—	5	7	20	48	65
Ammonia (mgm. per 10 ml.)	0.68	0.88	0.89	0.88	0.90	0.90	0.91	5.32
Residual histidine (mgm. per ml.)	2.15	2.15	2.15	2.15	1.95	1.90	1.68	0.523
Streptomycin production ('S' units per ml.)	—	—	—	—	—	40	230	80

The metabolism in the aspartic acid medium was characterized by a high production of mycelium, rapid uptake of glucose and histidine, ammonia production, increase in alkalinity, and a low yield of streptomycin. The lag phase was only about 24 hr. and then changes occur that are characteristic of rapid growth. The results obtained resemble those reported by Waksman

TABLE IV

METABOLIC CHANGES IN THE HISTIDINE – ASPARTIC ACID – ARGININE MEDIUM

Analysis of medium	Before sterilization	After sterilization	Duration of fermentation, days					
			1	2	3	4	5	7
pH	6.90	6.80	6.80	6.80	7.10	7.30	7.70	9.10
Residual glucose (mgm. per ml.)	12.2	12.4	12.2	11.3	9.4	6.0	1.25	0.25
Mycelium production (mgm. per 20 ml.)	—	—	—	11.5	29.1	55.5	85.0	80.0
Ammonia (mgm. per 10 ml.)	0.886	1.06	1.06	1.86	2.40	2.94	4.17	6.60
Residual histidine (mgm. per ml.)	2.15	2.15	2.15	1.88	1.10	0.55	0.06	0.02
Streptomycin production						10	18	30

and co-workers (13) using complex media. It can be concluded that the changes are all characteristic of growth and can not be correlated with the streptomycin producing mechanism. Histidine and glucose are consumed at approximately the same rate and practically disappear at the time mycelium production is maximal. The alkalinity increases steadily and is directly related to the corresponding rise in ammonia. Streptomycin production is low and bears no relation to mycelium production.

Up to the fifth day, the metabolism of the valine medium was characterized by less mycelium, slower uptake of glucose and histidine, practically no formation of ammonia, no change in hydrogen ion concentration but a high yield of streptomycin. The lag phase was of longer duration and although mycelium production was rapid from the third day on, the concentration of ammonia and the hydrogen ion concentration remained constant. It was not until the fifth day that the over-all changes resembled those in complex media. It is doubtful whether any of these factors are associated with the streptomycin producing mechanism. It can be tentatively concluded that the development of an alkaline reaction in complex media has no effect on the production of streptomycin.

It was possible to replace aspartic acid with glutamic or even with succinic acid plus nitrate and still obtain the same results as with aspartic acid, namely high mycelium but low streptomycin production. From these data, it is suggested that the metabolism of the aspartic acid, involving the aspartase–transaminase reaction, increases mycelium production at the cost of streptomycin, whereas another, as yet unexplained, mechanism operates in the case of valine. It appears as if the mycelium production does not parallel streptomycin production if the transamination systems are predominantly involved in the synthesis of mycelial protein.

It was shown that without sodium chloride Waksman's medium gave a low yield of streptomycin (8). Furthermore, all media so far developed that gave high streptomycin production contained sodium chloride (9, 3). One author reported that the chloride ion could be replaced by the sulphate ion, but the sodium ion could not be replaced by magnesium (7).

Two media were prepared, i.e., Waksman's medium with and without sodium chloride, and tested for streptomycin and mycelium production. The mycelium was separated by centrifuging and a difference in appearance was observed. Mycelium from the sodium chloride medium was dense in appearance, occupying only a small volume in the centrifuge tube, and had a dark color. The mycelium grown on the sodium chloride deficient medium was light colored, fluffy in appearance, and occupied a volume about two and one-half times that of the mycelium from the sodium chloride. When washed, dried, and weighed, however, the amount of mycelium produced in both cases was about the same. However, with the sodium chloride media the streptomycin content of the medium was higher.

A study of the metabolism of *Streptomyces griseus* in both media was carried out to elucidate the function of the high concentration of sodium chloride in Waksman's medium. The results with both media are given in one table, Table V, since there was no significant difference attributable to the influence of sodium chloride. However, as reported previously, the yield of strepto-

TABLE V

METABOLIC CHANGES IN WAKSMAN'S MEDIUM WITH AND WITHOUT SODIUM CHLORIDE

Period of fermentation	pH*	Streptomycin ('S' units per ml.)		Glucose (mgm. per ml.)*	Ammonia (mgm. per 10 ml.)*	Mycelium (mgm. per 10 ml.)*	Amino nitrogen (mgm. per 10 ml.)*
		With NaCl	Without NaCl				
0	6.9	—	—	9.5	0.15	—	2.4
1 day	7.5	—	—	9.0	0.29	28	2.0
2 days	7.5	130	25	5.0	0.27	104	1.0
3 days	7.9	180	30	1.7	0.30	82	1.1
4 days	8.5	180	70	0.8	0.47	64	0.9
5 days	8.7	130	130	0.8	0.50	49	1.0
6 days	8.8	80	80	0.7	0.52	42	1.0

**Values represent the average of both media.*

mycin and the physical consistency of the mycelia were not the same in the two media. The over-all destruction of streptomycin was rapid, either it was metabolized or inactivated at the alkaline reaction. A close relationship between the hydrogen ion concentration and the concentration of ammonia was again evident, apparently they are directly related. The α-amino nitrogen dropped to a minimum; apparently as proteolysis proceeded the free

amino acids were immediately utilized as shown by the accumulation of ammonia. In the sodium chloride deficient medium, lysis of the mycelium was accompanied by an increase in streptomycin in the medium, until equal to the sodium chloride medium.

This observation provided an explanation of the action of sodium chloride. As the concentration of salt increases in the medium the permeability of the membrane is affected in such a way as to cause an accumulation of streptomycin within the mycelium that is only liberated by lysis of the cell. In order to prove this hypothesis, mycelium was produced on Waksman's medium with and without sodium chloride and 10-ml. aliquots were transferred to centrifuge tubes. The medium was separated from the mycelium by centrifugation and the mycelium thoroughly washed by dispersing in water and recentrifuging. The mycelium of both media was treated as follows (Table VI).

TABLE VI

THE EFFECT OF SODIUM CHLORIDE IN WAKSMAN'S MEDIUM IN THE DISTRIBUTION OF STREPTOMYCIN BETWEEN MYCELIUM AND MEDIUM

Treatment of mycelium	Mycelium from medium without NaCl	Mycelium from medium with NaCl
	Zone of inhibition in mm.	
Tap water added, stored in refrig. (7 days)	27.0	21.0
0.5% NaCl added, stored in refrig. (7 days)	25.5	22.0
1% NaCl added, stored in refrig. (7 days)	26.0	22.0
0.1*M* phosphate, pH 7 added, stored in refrig. (7 days)	25.5	22.5
0.2*M* phosphate, pH 7 added, stored in refrig. (7 days)	26.5	22.0
Shaken with tap water 24 hr.	23.5	21.0
Shaken with 0.5% NaCl 24 hr.	26.0	23.5
Ground in mortar	26.5	22.5
Ground in mortar and mixture suspended in 0.1*M* phosphate buffer pH 7; stored in cold (7 days)	25.5	22.0

All volumes were kept constant and the results are comparable. Excellent agreement in the amount of streptomycin produced was obtained by mechanical rupture of the mycelium, as compared to shaking in saline or slow diffusion at 4° C. for seven days. From these results it seems evident that streptomycin synthesis does not occur when the metabolic activity is at a minimum.

An exact study of the daily variations in the distribution of streptomycin between mycelium and media was next carried out (Table VII). There appears to be a limit to the amount of streptomycin synthesized because the total production is constant; however, the distribution varies with salt concentration and duration of fermentation. As lysis proceeds, the streptomycin is liberated from the mycelium and is destroyed at the same rate in both media. The antibiotic does not seem to be metabolized but is most probably inactivated by the accumulation of alkali.

TABLE VII

STREPTOMYCIN DISTRIBUTION BETWEEN MYCELIUM AND MEDIA—WAKSMAN'S MEDIUM WITH AND WITHOUT SODIUM CHLORIDE

Days	Medium with NaCl		Medium without NaCl	
	Zone of inhibition (mm.)			
	Mycelium	Medium	Mycelium	Medium
1	11.0	23.0	21.0	23.5
2	23.0	25.5	26.0	21.0
3	22.0	26.5	25.0	22.5
4	20.0	25.5	20.0	25.0
5	19.0	24.5	19.0	24.5

Since it has been reported (7) that some ions can replace sodium chloride, whereas others can not, an experiment was carried out in which the sodium or chloride ion was replaced by other cations or anions. Little or no difference was observed in the cation series. With the anions a more selective action was observed. This was apparent, not only in the distribution of streptomycin but also in the type of mycelium produced. With tartrate the mycelium was dense and dark in color, while with the thiocyanate it appeared fluffy, swollen, and light in color. This was proof that the action of the sodium of chloride ion was not specific but that the anion and cation could be replaced by others, provided they were close to the sodium or chloride ion in the Hofmeister lyotropic series.

These observations suggest the possibility of developing new methods for the isolation of streptomycin. Rather than recovering the antibiotic from a deeply colored, highly complex medium, it might be easier to isolate it from the mycelium. As Molitor (6) pointed out, "fundamental changes will have to be brought about in the present isolation and purification technique in order to obtain streptomycin concentrates not contaminated with biologically active substances."

Summary and Conclusion

Amongst the amino acids, histidine alone serves as a readily available nitrogen source that promotes both streptomycin and mycelium production. Valine, however, plays a more specific role, it promotes streptomycin synthesis only, provided other factors are present that will promote the formation of mycelium. The addition of aspartic acid definitely inhibits synthesis of the antibiotic but accelerates production of mycelium and induces similar metabolic changes in the medium as does a complex organic medium. Since aspartic, glutamic, or succinic acid exhibit a similar effect upon the organism, it may be concluded that the synthesis of mycelium proceeds by the aspartase–transaminase system but this inhibits streptomycin formation. However, another mechanism, as yet unexplained, will also bring about the synthesis of mycelial

protein and apparently there is some connection between this alternate path of mycelial synthesis, the role of valine, the action of inositol, and the production of streptomycin.

The influence of high concentrations of sodium chloride can not be ascribed to osmotic pressure effects. It appears more likely, in view of the observations with the ions of the lyotropic series, that the permeability of the membrane is affected by the concentration as well as the nature of the ion. These observations also suggest that the antibiotic is a product of intracellular synthesis and diffuses into the medium when the salt concentration is high. Unexplained, however, is the observation that on the second day of fermentation in a medium without added sodium chloride, more streptomycin is found in the medium than in the mycelium.

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SOME FACTORS INFLUENCING THE GROWTH AND SURVIVAL OF RHIZOBIA IN HUMUS AND SOIL CULTURES[1]

By R. A. Hedlin[2] and J. D. Newton[3]

Abstract

A study was made of the growth of rhizobia outside the host plant in three high humus mixtures and a mineral soil. Rhizobia grew better in pure cultures than in an unsterile base. Incubation in Erlenmeyer flasks gave, as a rule, higher counts than in paper cartons. Addition of a mixture of sucrose, calcium carbonate, sodium chloride, dipotassium phosphate, and magnesium sulphate led to increased numbers of rhizobia in pure culture in all bases used, and produced increased numbers in the unsterilized mineral soil also. Addition of this mixture to the unsterile high humus bases increased the total numbers of bacteria, but not the number of rhizobia. When the bases were sterilized, the highest counts of rhizobia were obtained in the high humus bases. When sterilization was not carried out, the greatest numbers of rhizobia occurred in the mineral soil. In tightly stoppered flasks that did not permit aeration, the rhizobia appeared to die out completely when stored for more than a few days, whereas in loosely stoppered flasks the counts remained at a high level until the base became desiccated.

Introduction

To obtain the optimum growth of leguminous crops, inoculation of the seed with legume bacteria is frequently necessary. When inoculating legume seed, there are two important factors that must be considered. First the inoculant applied to the seed should contain large numbers of bacteria of the cross-inoculation group in question, and second, these bacteria should be of a strain that is efficient in fixing nitrogen in association with the inoculated legume. It was with the first of these problems in mind that this study was initiated. The effects of aeration, moisture content, and addition of nutrient salts on the growth of rhizobia in humus and soil cultures were investigated.

Review of Literature

The first use of soil as a base in which to grow legume bacteria was reported by Simon (22). Since moist soil cultures were first introduced, their use has increased widely. Temple (24) also has reported on steam sterilized soils as a medium for growing rhizobia. He compared the numbers of rhizobia per gram of soil with the numbers in a nutrient solution and found the latter very inferior as a medium in which to grow the organisms. Similar results were obtained using legume bacteria from crimson clover and alfalfa. The soil used by Temple was low in fertility, and he found that by the addition of various organic compounds such as alfalfa meal, sucrose, etc., he was able to increase the numbers of bacteria very markedly. In this work, however, counts were not continued beyond the 42nd day.

[1] *Manuscript received January 20, 1948.*

Contribution from Department of Soils, University of Alberta, Edmonton, Alta., with financial assistance from the National Research Council of Canada.

[2] *Graduate Assistant in Soils, University of Alberta. Now Graduate Assistant in Agricultural Bacteriology, University of Wisconsin, Madison, Wis.*

[3] *Professor of Soils, University of Alberta.*

In 1936 Hofer (12) made a study of the numbers of rhizobia in humus cultures. He reported that the numbers were highly variable. After one week the count was usually higher than immediately after adding culture to humus base, but after 50 days it was lower. After one year the count of nodule bacteria was only 1% of what it had been immediately after inoculation.

Fellers (5) carried out extensive experiments on commercial cultures of legume inoculants. In his work he studied the numbers of bacteria in the various inoculants as well as their ability to cause infection of the host plant. He found that the numbers of bacteria varied greatly. In general those cultures with less than one million organisms per gram were not effective, although it was impossible to set definite limits. He found that cultures that gave high plate counts usually gave satisfactory nodulation of legume plants.

The importance of free access to air on the growth and survival of rhizobia has been emphasized by a number of workers including Alicante (1), Gangulee (10), Fred *et al.* (8), and Ockerblad (18). There are, however, reports (Edwards (4) and Jones (13)) that indicate that the rhizobia are able to survive for many years when stored in sealed containers.

Pitz (20) noted the beneficial effect of small amounts of calcium sulphate on the growth of pure cultures of rhizobia. Truesdell (26) observed that a small amount of added phosphorus increased the number of alfalfa nodule bacteria in sterilized soils. Fulmer (9) reported that the addition of calcium carbonate or magnesium carbonate greatly stimulated the growth of rhizobia from alfalfa or lupines in sterilized acid soil. Thornton (25) and Alicante (1) also stress the importance of calcium on the growth of legume bacteria outside the host plant.

Like all nonsporeforming bacteria, the rhizobia are sensitive to excessive drying. The effect of desiccation, however, depends on several conditions—the nature of the substrate, reaction, temperature, etc. Giltner and Langworthy (11) carried out extensive studies on the longevity of rhizobia under various moisture conditions. They found that rhizobia survive a long time in air-dry soil, probably because of the films of hygroscopic moisture that surround the particles. Vandecaveye (27) and Temple (24) have likewise reported on the remarkable viability of rhizobia in air-dried soil.

Another factor that appears to influence the growth of rhizobia in soil is the symbiotic or antagonistic effect of other microorganisms. As early as 1899, Nobbe and Hiltner (17) suggested that the competition between rhizobia and the rest of the soil flora results in harm to the rhizobia. They implicated both bacteria and fungi in the opposition. Sackett (21), in a laboratory study of associated growth of *B. ramosus* and rhizobia, observed that multiplication of the latter is greatly inhibited. Duggar and Prucha (3) obtained better growth of rhizobia in sterilized than in unsterilized soil, and argued that the general soil flora have an antagonistic action towards rhizobia.

At Wisconsin Konishi (14) obtained some interesting data on the relation of certain soil bacteria to rhizobia. He isolated several unidentified organisms from the soil and tested them with rhizobia in a liquid medium, with and without calcium carbonate. In general, antagonistic effects of other microorganisms towards rhizobia were most severe in liquid media without calcium carbonate and least severe in soil.

There are also reports of beneficial effects of certain organisms upon rhizobia. Bottomley (2) reported enhanced nitrogen fixation by mixed cultures of nodule bacteria and *Azotobacter*. Manns and Goheen (16) and Fellers (5) referred to a beneficial effect from the association of *Azotobacter* and rhizobia upon colony formation in Petri plates. Löhnis and Hansen (15) also noted the stimulating effect of *B. radiobacter* upon colony formation of the cowpea–soybean cross-inoculation group.

Outline of Investigation

In this work four different inoculant bases or media were used for culturing the rhizobia.

These materials were:

1. Winterburn peat. This is a high lime peat obtained in a bog at Winterburn, which is several miles west of Edmonton. The peat used was fairly well decomposed material obtained at a depth of approximately four feet.

2. Peat – black soil mixture. This base was prepared by mixing equal portions by weight of the base described above with a black mineral soil from the University plots just south of Edmonton.

3. Peat – carbon black mixture. This inoculant base consisted of a fine, well-decomposed peat obtained near Edmonton. The peat was mixed with a small amount of carbon black. This material was obtained through the courtesy of Mr. J. A. Robertson of Edmonton, who had used it as an inoculant base in preparing commercial cultures of rhizobia.

4. Edmonton loam. This is a black zone soil from the University plots just south of the city of Edmonton. This soil comprised one-half of inoculant base number two.

Cultures of *R. trifolii* and *R. leguminosarum* were stored in the four bases listed above. Containers holding the various bases were inoculated with cultures of these two organisms and were stored at room temperature under a variety of conditions. Plate counts of the bacteria were made at intervals during the storage period—usually at 10, 40, 70, and 140 days after inoculation.

The plan of the experiments is shown in Table I. As indicated in Table I, storage was carried out in paper cartons for two of the experiments, IV and VII. In the other experiments storage was carried out in Erlenmeyer flasks. Where cartons were used, they were waxed over at the beginning of the storage period to prevent too rapid a loss of moisture. When storage was carried out in Erlenmeyer flasks, some of the flasks were tightly stoppered, whereas others were stoppered loosely so that a free exchange of air was possible. To stopper

TABLE I

PLAN OF EXPERIMENTS ON THE GROWTH AND SURVIVAL OF *R. leguminosarum* (EXPTS. I TO IV, TABLES II TO V) AND *R. trifolii* (EXPTS. V TO VII) IN HUMUS AND SOIL CULTURES

	Bases used	Stopperings	Treatments		Storage periods, days
Expt. I	Peat Peat – black soil Peat–carbon mixture Black soil	Tightly stoppered Cotton stoppered	Sterile and unsterile	No nutrients added	0 10 38 72 148
Expt. II	Same as above	All cotton stoppered	All sterile	Nutrients added to half of flasks	0 14 42 90 141
Expt. III	Peat–carbon mixture Black soil	All cotton stoppered	All unsterile	Nutrients added to half of flasks	0 12 25 70 112
Expt. IV	Peat Peat – black soil Peat–carbon mixture Black soil	Waxed paper cartons	Sterile and unsterile	No nutrients added	0 11 48 77 146
Expt. V	Same as Expt. I except *R. trifolii* used				0 14 38 70 142
Expt. VI	Same as Expt. II except *R. trifolii* used				0 20 48 89 140
Expt. VII	Same as Expt. III except *R. trifolii* used				0 8 36 68 146

flasks tightly, rubber stoppers waxed over with paraffin were used. In stoppering flasks loosely, on the other hand, the practice was to use a rubber stopper one size smaller than would normally have been used, and to wrap a little absorbent cotton around it. This allowed for a free exchange of air in the Erlenmeyer flask, but the drying out was less rapid than would have been the case had an ordinary cotton stopper been used.

In those cases where the inoculant base was supplemented by the addition of nutrients, the following mixture was used:

Sucrose	— 100 gm.
Dipotassium hydrogen phosphate	— 10 gm.
Sodium chloride	— 2 gm.
Magnesium sulphate	— 4 gm.
Calcium carbonate	— 60 gm.

These salts and sucrose were used in the proportions given above in the media recommended by Fred *et al.* (6) and by Fred and Waksman (7) for plating legume bacteria. It was added to the inoculant base at the rate of 1.4% by weight.

In a number of the experiments the growth and survival of pure cultures of rhizobia were studied. In such cases the humus bases were sterilized in the autoclave at 15 lb. steam pressure (121° C.). The peat, peat – black soil mixture, and the peat–carbon mixture were held at this temperature for two and a half hours, while the black soil was heated in the autoclave at 121° C. for 25 min. Simpson (23) found that this treatment gave complete sterilization of these bases in the amounts used here.

Inoculations were made by using a suspension of bacteria in a 0.75% sodium chloride solution. To make up this suspension bacteria were grown on agar slants in Erlenmeyer flasks. After a good growth had been obtained the sodium chloride solution was added, the growth scraped free from the surface of the agar with a sterile inoculating needle, and a suspension formed by rotating the flask to get an even distribution of the bacteria. This suspension was added to the inoculant bases at the rate of 10 cc. per 50 gm. of base. Counts of the number of legume bacteria were made at the time of inoculation. From this, the initial count of legume bacteria per gram of base could be calculated. All counts were made by the plate method using as the medium yeast extract – sucrose agar containing Congo red, as recommended by Fred *et al.* (6) and by Fred and Waksman (7). The Congo red aids in the detection of contaminants that might be present. Detection is possible since most of the contaminants absorb the dye, thus producing reddish colonies, whereas the colonies of legume bacteria remain clear.

At the beginning of the storage periods all bases were made up to 30% of their total water holding capacities. This 30% included the water carrying the suspension of nodule bacteria. The tightly stoppered flasks remained at this water content throughout the storage period. Weighings of the flasks at the beginning and at the end of the storage period indicated that there was no moisture loss from these flasks. All water percentages given in the tables are expressed on the air-dry basis.

Results

The results of Expts. I to IV, including bacterial counts and moisture percentages in the different inoculant bases are given in Tables II to V. All counts are in millions per gram and the incubation times and treatments are indicated in the tables. The counts and moisture percentages are on the air-dry basis.

The counts of bacteria in the inoculant bases studied in this investigation are very closely correlated with the moisture content and aeration of the inoculants. This is particularly true of the counts of rhizobia, but is also very noticeable in the total counts of unsterilized inoculant bases.

TABLE II

EFFECT OF VARIOUS TREATMENTS ON PLATE COUNTS OF *R. leguminosarum* (B_3) AND OTHER MICROORGANISMS IN FOUR INOCULANTS—PEAT, PEAT – BLACK SOIL MIXTURE, PEAT–CARBON MIXTURE, AND BLACK SOIL (MILLIONS PER GRAM)

Storage time in days	Sterile base						Unsterile base					
	Stoppering											
	Rubber			Cotton			Rubber			Cotton		
	Nodule bacteria count	Total count	Water, %	Nodule bacteria count	Total count	Water, %	Nodule bacteria count	Total count	Water, %	Nodule bacteria count	Total count	Water, %
Peat (no nutrients added)												
0	30.4	30.4	137.4	30.4	30.4	137.4	30.4	32.9	137.4	30.4	32.9	137.4
10	181.5	181.5	137.4	285.0	285.0	134.2	16.0	1011.0	137.4	15.0	819.0	135.0
38	0.0	0.0	137.4	129.0	129.0	127.4	0.0	224.0	137.4	12.0	1207.0	124.0
72	0.0	0.0	137.4	31.7	31.7	117.0	0.0	203.0	137.4	5.0	589.0	117.4
148	0.0	0.0	137.4	10.6	10.6	101.8	0.0	148.0	137.4	0.0	449.0	107.4
Peat – black soil mixture (no nutrients added)												
0	30.4	30.4	81.0	30.4	30.4	81.0	30.4	31.9	81.0	30.4	31.9	81.0
10	292.0	292.0	81.0	341.0	341.0	78.8	11.5	275.0	81.0	3.3	416.5	78.8
38	0.2	0.2	81.0	190.0	190.0	72.4	0.0	205.0	81.0	7.0	261.0	71.0
72	0.0	0.0	81.0	238.0	238.0	57.6	4.0	116.0	81.0	5.5	195.0	63.2
148	0.0	0.0	81.0	50.0	50.0	42.0	0.0	158.0	81.0	0.0	131.0	50.2
Peat – carbon mixture (no nutrients added)												
0	30.4	30.4	69.2	30.4	30.4	69.2	30.4	100.2	69.2	30.4	100.2	69.2
10	490.0	490.0	69.2	570.0	570.0	67.2	20.0	396.5	69.2	8.8	784.0	67.4
38	0.0	0.0	69.2	160.0	160.0	60.8	0.0	101.0	69.2	9.0	921.2	62.6
72	0.0	0.0	69.2	376.0	376.0	50.8	0.0	193.0	69.2	0.8	617.0	53.2
148	0.0	0.0	69.2	62.0	62.0	27.8	0.0	507.0	69.2	0.0	401.2	34.2
Black soil (no nutrients added)												
0	30.4	30.4	29.2	30.4	30.4	29.2	30.4	30.9	29.2	30.4	30.9	29.2
10	265.0	265.0	29.2	303.0	303.0	27.6	58.0	58.0	29.2	50.0	55.0	28.0
38	60.0	60.0	29.2	258.0	258.0	20.2	98.0	183.0	29.2	287.0	454.0	22.0
72	0.0	0.0	29.2	142.0	142.0	16.6	3.3	10.6	29.2	1.6	6.4	15.0
148	0.0	0.0	29.2	0.0	0.0	5.0	2.9	11.2	29.2	0.0	59.0	6.6

The results of Expt. I in Table II show very definitely that in all four inoculant bases used, the numbers of rhizobia drop sharply when stored in sealed flasks. This is particularly noticeable in the peat, peat – black soil, and peat–carbon mixtures. In these tightly stoppered sterile bases the numbers of rhizobia rose for a time, being considerably higher when the second plate count was made after storage for 10 days than they had been at the time

of inoculation. In the third and subsequent counts in these three bases, the numbers had dropped below 50 thousand per gram.

In the fourth base, black soil, at 38 days the count of rhizobia in tightly stoppered flasks was still higher than at the beginning of the storage period. However, in this base the numbers of rhizobia had dropped below 50 thousand per gram by the time the fourth count was made 72 days after the beginning of the storage period.

In the unsterile, tightly stoppered flasks, results somewhat similar to those with sterile bases were obtained. In the peat, peat – black soil, and peat–carbon mixture, when the third count was made after storage for 38 days, no typical colonies of rhizobia appeared on the plates. There was a slight discrepancy in the peat – black soil mixture where a count of four million rhizobia per gram was obtained in the fourth count at 72 days, but this had again dropped below 50 thousand per gram when the fifth count was made. An interesting fact brought out here is that, unlike the counts in the sterile containers, when the second count was made 10 days after the beginning of the storage period, the count of rhizobia had dropped far below the initial numbers. This would seem to indicate that the report made by Fellers (5) that it is unnecessary to sterilize peat or soil when used as a base for culturing rhizobia is incorrect. The presence of other soil organisms seems to exert a very strong antagonistic effect upon the growth of rhizobia. This is indicated by the fact that there was a high total count coupled with these low counts of rhizobia.

In the unsterile, tightly stoppered flasks the total count rose quickly from the beginning of the storage period until the second count was made at 10 days, and then fell quite sharply in the succeeding counts. In the peat – black soil mixture and in the peat–carbon mixture the plate counts indicated a rise in the total numbers of bacteria between the fourth and fifth counts. This may have been due to errors in the plate counts or merely to a variation in the different flasks. In the unsterile tightly stoppered flasks containing black soil, the total count of bacteria did not rise as rapidly as in the other three inoculants. However, the count of rhizobia did rise quite markedly in this base, reaching 98 million per gram after 38 days' storage. The number of rhizobia as well as the total number of bacteria had dropped quite sharply in this base by the time the fourth and fifth counts were made.

In the cotton stoppered, sterile flasks, the counts in all bases had risen far above the number initially introduced when the second count was made. A marked drop was evident when the third count was made after 38 days' storage. A slight rise took place in the peat–carbon and peat – black soil mixtures between the third and fourth counts, while in the peat and in the black soil the numbers of rhizobia continued to drop. When the fifth count was made after 148 days' storage, no rhizobia were present in the black soil, while the peat–carbon mixture had the highest count, 62 millions per gram.

In the unsterile, cotton stoppered flasks as in the unsterile, tightly stoppered flasks, the numbers of rhizobia had dropped sharply in all bases except the

black soil, when the second count was made. The number of rhizobia in the peat – black soil mixture rose slightly between the second and third counts but fell again thereafter. In all of these bases no rhizobia were present when the final counts were made after 148 days' storage. Once again, as in the tightly stoppered flasks, counts of rhizobia and other bacteria in the black soil were higher after 10 days' storage than they had been initially. The number of rhizobia had risen still higher after 38 days' storage. Numbers of rhizobia dropped quite sharply between the third and fourth counts and when the fifth count was made after 148 days' storage none were found.

The total counts in all four bases in unsterile cotton stoppered flasks showed, in general, a sharp rise, followed by a fall in numbers. In peat, peat–carbon, and black soil the largest numbers of bacteria were counted after 38 days' storage, while in the peat – black soil mixture the largest number was counted after 10 days' storage.

The counts of rhizobia given in Table III (Expt. II) show that where nutrients are added to the inoculant bases there is a marked increase in the numbers of nodule bacteria. The only exceptions to this are in the fourth counts of the numbers of rhizobia in the peat and the peat – black soil mixture. Here the number of rhizobia in the base to which no nutrients were added was higher than where nutrients were added. As in Table II, the results indicate the importance of moisture content on the numbers of rhizobia. The numbers rose for a time, but as the percentage of moisture in the base fell, the numbers of bacteria dropped quite markedly as well. The greatest numbers were usually present at the second count (14 days) but sometimes at the third (42 days). The most marked increases in numbers of legume bacteria resulting from the addition of nutrients were obtained in the peat – black soil and peat–carbon mixtures. With no nutrients added, the counts of rhizobia in the peat–carbon mixture were considerably higher than in the peat – black soil mixture. Another interesting point in the data of this table is that the counts of rhizobia in the peat–carbon mixture treated with nutrients remained very high for 90 days. In all the other treatments, the numbers had dropped to a fraction of their highest count by this time.

A comparison of the counts in flasks to which no nutrients were added, in Table III, with those similarly treated in Table II, shows that these counts follow similar trends. In both cases the numbers increased for a time and then dropped sharply as the bases dried out.

Table IV gives the results of Expt. III. It was thought that it might be more practical, if possible, to use unsterilized inoculant bases. Therefore, in this experiment a study was made of the growth of rhizobia in unsterile inoculant bases with and without added nutrients.

In the peat–carbon mixture there seems to be no significant difference between counts of rhizobia in the base to which nutrients were and were not added. There was, however, a definite increase in the total number of bacteria

TABLE III

PLATE COUNTS OF *R. leguminosarum* (B_8) IN THE FOUR INOCULANTS STORED IN ERLENMEYER FLASKS, STERILIZED AND STOPPERED WITH COTTON (MILLIONS PER GRAM)

Storage time in days	Nutrients added		No nutrients added	
	Nodule bacteria count	Water, %	Nodule bacteria count	Water, %
Peat				
0	63.3	137.4	63.3	137.4
14	646.0	134.0	286.0	134.2
42	138.0	125.2	114.0	127.6
90	44.0	110.6	53.0	113.8
141	28.7	95.6	10.5	89.6
Peat–black soil mixture				
0	63.3	81.0	63.3	81.0
14	1794.0	77.0	526.0	78.6
42	1585.0	73.6	350.0	71.0
90	208.0	57.4	216.0	53.2
141	97.5	46.6	40.9	39.0
Peat–carbon mixture				
0	63.3	69.2	63.3	69.2
14	1997.0	65.4	798.0	66.4
42	1617.5	59.2	918.0	56.6
90	1678.0	40.0	364.0	40.2
141	291.0	31.4	176.0	23.8
Black soil				
0	63.3	29.2	63.3	29.2
14	578.0	24.4	337.5	25.0
42	676.2	20.8	352.0	19.0
90	26.0	3.8	4.9	4.2
141	3.1	0.8	1.3	0.0

in the base to which nutrients were added. This suggests that the competition of additional soil bacteria must have offset the beneficial effect derived by rhizobia from the nutrients added. In the black soil the addition of nutrients gave a very definite increase in numbers of rhizobia as well as in total count, over the base to which no nutrients had been added. In the soil to which no nutrients were added, the numbers of rhizobia did not increase above the initial number added. Where nutrients were added, after 12 days' incubation, the number of rhizobia had risen to approximately double the initial count. This is in marked contrast with the counts of rhizobia in the

TABLE IV

PLATE COUNTS OF *R. leguminosarum* (B_3) IN TWO INOCULANTS, STORED IN ERLENMEYER FLASKS, UNSTERILE AND STOPPERED WITH COTTON (MILLIONS PER GRAM)

Storage time in days	Nutrients added			No nutrients added		
	Nodule bacteria count	Total count	Water, %	Nodule bacteria count	Total count	Water, %
Peat–carbon mixture						
0	101.2	181.0	69.2	101.2	181.0	69.2
12	15.0	1189.0	64.4	32.0	741.0	64.2
25	11.0	1021.0	62.0	9.0	425.0	54.8
70	4.0	541.0	41.4	5.0	406.0	48.4
112	0.1	512.0	32.4	0.4	308.0	35.2
Black soil						
0	101.2	101.7	29.2	101.2	101.7	29.2
12	198.0	296.0	25.2	87.0	127.0	25.2
25	168.0	371.0	21.4	92.5	162.5	22.8
70	5.5	95.0	8.8	1.0	22.5	14.2
112	13.0	90.0	5.8	0.5	2.3	5.6

peat–carbon mixture, where after 12 days' incubation, the number of rhizobia present had dropped to a fraction of those originally present.

Table V represents the results of Expt. IV and contains the bacterial counts for inoculants stored in paper cartons. It includes counts for both sterile and unsterile inoculant bases. In the paper cartons containing sterilized inoculant base, some recontamination did take place. However, as the total counts were not recorded, it is impossible to say what effect this might have had on the numbers of rhizobia. In the sterilized bases the highest counts were obtained after 11 days' storage. The numbers were still fairly high after 77 days, but were quite small after 146 days, and in one case they had disappeared entirely after 146 days.

A comparison of the data in this table with that in Tables II and III indicates that the numbers of rhizobia in sterile bases stored in cartons do not rise as high as in similarly treated bases stored in Erlenmeyer flasks. The reason for the Erlenmeyer flasks being better containers in which to culture rhizobia seems hard to determine. There are several factors that may have been contributory. Although total counts were not recorded, it was noticed that in many of the sterilized bases stored in cartons appreciable mold growth had taken place on the surface of the base. The mold may have had an inhibitory effect on the growth of the rhizobia. The paper cartons, in spite of being waxed, usually retained a lower percentage of moisture at the end of the storage period than did the bases stored in cotton stoppered flasks. This is another

TABLE V

PLATE COUNTS OF *R. leguminosarum* (B_3) IN FOUR INOCULANTS, STERILE AND UNSTERILE, STORED IN PAPER CARTONS (MILLIONS PER GRAM)

Storage time in days	Sterile			Unsterile		
	Nodule bacteria count	Total count	Water, %	Nodule bacteria count	Total count	Water, %
Peat (no nutrients added)						
0	35.8	35.8	137.4	35.8	38.3	137.4
11	134.0	—	134.2	8.0	908.0	130.4
48	46.0	—	128.6	13.0	945.0	105.0
77	72.0	—	114.0	1.0	754.0	102.6
146	4.3	—	86.6	0.0	541.0	63.2
Peat–black soil mixture (no nutrients added)						
0	35.8	35.8	81.0	35.8	37.3	81.0
11	210.0	—	77.8	14.0	277.5	78.6
48	107.5	—	69.0	6.0	271.0	62.6
77	103.0	—	59.4	3.0	229.0	63.4
146	0.0	—	30.6	0.0	130.0	30.2
Peat–carbon mixture (no nutrients added)						
0	35.8	35.8	69.2	35.8	115.6	69.2
11	349.0	—	66.8	14.0	878.0	64.6
48	284.0	—	58.6	3.8	853.8	53.4
77	199.0	—	48.8	1.0	267.0	26.8
146	11.6	—	20.4	1.0	391.0	22.0
Black soil (no nutrients added)						
0	35.8	35.8	29.2	35.8	36.3	29.2
11	147.0	—	27.4	3.0	13.3	25.8
48	45.0	—	22.4	0.2	10.0	16.2
77	21.5	—	18.8	0.7	7.7	15.4
146	1.0	—	9.6	0.2	8.3	9.2

factor that could have contributed to the lower count of rhizobia in the bases stored in cartons. However, it is questionable whether this could account for all the difference since in the early stages of storage there was usually little difference between the moisture contents of the bases in the cartons and those in the flasks. A third possible factor is the difference in the initial number of bacteria introduced into the bases in the various experiments. In view of the rapid multiplication of bacteria under favorable conditions, it seems questionable whether this was of importance.

In the unsterilized bases stored in paper cartons, a similar trend to that obtained in unsterilized bases stored in Erlenmeyer flasks is evident. Here

TABLE VI

STORAGE PERIODS BETWEEN WHICH *R. leguminosarum* FELL FROM OVER ONE MILLION PER GRAM TO LESS THAN ONE MILLION PER GRAM

Treatment	Peat	Peat – black soil mixture	Peat–carbon mixture	Black soil
	Days			
Sterilized flasks, no nutrients added, tightly stoppered	10 – 38	10 – 38	10 – 38	38 – 72
Sterilized flasks, no nutrients added, cotton stoppered	More than 148	More than 148	More than 148	72 – 148
Unsterilized flasks, no nutrients added, tightly stoppered	10 – 38	10 – 38	10 – 38	More than 148
Unsterilized flasks, no nutrients added, cotton stoppered	72 – 148	72 – 148	38 – 72	72 – 148
Sterilized flasks, nutrients added, cotton stoppered	More than 141	More than 141	More than 141	More than 141
Sterilized flasks, no nutrients added, cotton stoppered	More than 141	More than 141	More than 141	More than 141
Unsterilized flasks, nutrients added, cotton stoppered	—	—	70 – 112	More than 112
Unsterilized flasks, no nutrients added, cotton stoppered	—	—	70 – 112	70 – 112
Sterilized cartons, no nutrients added	More than 146	77 – 146	More than 146	146*
Unsterilized cartons, no nutrients added	77 – 146	77 – 146	146*	11 – 48

— *Not determined.*

* *Count of one million per gram at 146 days.*

the tendency was to obtain high total counts coupled with low counts of rhizobia. As in other counts of rhizobia in unsterilized inoculant bases, the numbers of rhizobia had dropped quite sharply by the time that the second plate counts were made after about 11 days' storage. They continued to drop in subsequent plate counts. When the final counts were carried out in the peat and peat – black soil mixtures after 146 days' storage, no rhizobia were present, while in the peat – carbon black mixture and the black soil, the numbers were 1.0 and 0.2 millions per gram respectively. The total count in the unsterilized bases rose quite high except in the black soil, but dropped off gradually as the inoculant base dried out.

Experiments were also carried out using red clover bacteria instead of pea bacteria. These experiments were, in all other respects, identical with Expts. I, II, and IV reported in Tables II, III, and V. Since the results were very similar to those obtained with pea bacteria, the data have not been included.

It is realized that it would have been desirable to supplement the plate counts of rhizobia in the inoculants with cultural experiments. However, time and facilities did not permit the carrying out of such work. Nevertheless Table VI has been set up to indicate the length of time for which the various inoculant bases remained effective. This is based on results reported by Fellers (5). He found that cultures that contained more than one million rhizobia per gram usually were effective in producing nodules on legume plants when applied to the seed before planting. Such a criterion of effectiveness of cultures is, however, merely a guide.

The data indicate, on the basis of this criterion, that all of the tightly stoppered sterilized and unsterilized inoculants except black soil remained effective for less than 38 days, black soil inoculants remaining effective for somewhat longer periods. In the sterilized cotton stoppered flasks all of the inoculants except black soil remained effective for more than 148 days, black soil remaining effective for more than 72 but less than 148 days. In another experiment all of the previously sterilized cotton stoppered flask inoculants, with and without the addition of nutrients, remained effective for more than 141 days. In the unsterilized cotton stoppered flasks all of the inoculants remained effective for more than 72 days, but less than 148 days, except the peat–carbon mixture, which remained effective for more than 38 days but less than 72.

In addition to counts of bacteria, the inoculant bases were analyzed for available phosphorus, potassium, calcium, nitrate nitrogen, and ammoniacal nitrogen. This was done by using the method of Peech and English (19). It was hoped from this work that it would be possible to correlate the amounts of the above-mentioned ions present with the growth of rhizobia in the bases. However, the method of analysis was not always sensitive enough to reflect the small differences in chemical composition brought about by differences in treatment. It did indicate that sterilization of high humus bases increased the content of ammoniacal nitrogen very markedly. Addition of the nutrient mixture containing sucrose, calcium carbonate, dipotassium phosphate, sodium chloride, and magnesium sulphate was usually reflected by increased amounts of potassium and phosphorus as shown by this method. Additions of calcium were not usually reflected by this method of analysis. Similarly, treatment differences such as sterilization, length of incubation, etc. did not show any consistent difference in chemical composition as determined by this method. Thus it was impossible to make the desired correlation between availability of these ions and the growth of the rhizobia.

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FLUORESCENCE MICROSCOPE EXAMINATION OF BACTERIA IN SOIL[1]

By S. Strugger[2]

Abstract

By the staining of soil with the fluorescent dye acridinorange and examination of the stained soil under the fluorescence microscope, it is possible to observe directly the living bacteria of soil in their autochthonic condition. All humus substances are stained red in color. The bacteria, living on the humus particles, are of green fluorescence and give an excellent contrast against the red background. The bacteria are vitally stained and can be used for culture experiments. Using this method in some soil tests the autochthonic bacteria of soil have been investigated qualitatively and quantitatively.

In microbiological research on soil, two methods may be used.

1. With the aid of culture plates the organisms are isolated from the soil and identified. In general, all microscopical examinations of the soil have been made by this method (on which the hitherto existing results of microbiological results are therefore based) but they do not permit any conclusion about the autochthonic condition of the soil bacteria. Quantitative determination of the bacteria, too, has been performed in this way. The results do not stand critical examination, however, because not all soil microorganisms can grow on the culture media used or can produce countable colonies.

2. Conn (1918 (1), 1929 (2)) and Winogradsky (1925 (6, 7), 1928 (8)) tried to observe the bacteria of soil by direct microscopical examination of stained smears. Until now, however, this method of direct microscopical analysis has not been very successful because, with the stain ordinarily used, the bacteria and the particles of soil are stained the same color. Therefore a distinction between the particles of soil and the bacteria is possible only under the most favorable conditions. There is no doubt that the direct microscopical examination of the autochthonic soil bacteria is a desirable objective of the microscopical research of soil that could not be reached until now because of technical difficulties.

In this paper will be given information about a new method of vital staining of soil bacteria with the fluorescent dye, acridinorange; this permits an analysis of the autochthonic bacteria of soil by means of the fluorescence microscope.

General Remarks about the Acridinorange Method

In 1940, Strugger (3) succeeded in solving the problem of the vital staining of cytoplasm and nucleus of plant cells with the aid of the fluorescent dye,

[1] *Manuscript received in original form January 17, 1947, and as revised, July 2, 1947.*
Contribution from the Botanical Institute of the Veterinary College of Hannover, Hannover, Germany.

[2] *At the time, Professor of Botany and Director of the Botanical Institute of the Veterinary College of Hannover. Now, Director of the Botanical Institute and Botanical Garden of the University of Münster, i. W., Schlossgarten, Germany.*

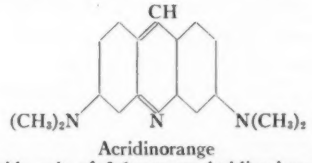
acridinorange (3,6-tetramethyldiaminoacridin).* The albuminoid components and nucleoprotein of the living protoplasm have an extraordinary affinity for this dye. As the fluorescence microscope method of observation lies, in sensitivity, between perception by smelling and electroscopical detection of radioactivity, only an extremely small absorption of the dye in protoplasm is necessary. Therefore the harmful effect of the vital staining is so restricted that the vitally stained protoplasm of plant cells can continue to grow.

The fluorescent dye, acridinorange, is a basic dye. It is dissociated in the whole of the acid, the neutral, and the basic region up to pH 8.5.

The acridinorange cations show a variable fluorescence according to the concentration. Dissociated solutions of lower concentration (1 : 100,000 to 1 : 10,000) have green fluorescence, whereas concentrated solutions (1 : 100) shine in copper-colored fluorescence under the quartz lamp.** The center of gravity of the fluorescence spectrum of weak aqueous solutions of acridinorange is displaced, reversibly, with rising concentration, to the region of long waves. This 'effect of concentration', investigated by Strugger (1940 (3)), is of great importance in the use of acridinorange as a vital stain.

If living plant cells are vitally stained by acridinorange (solution 1 : 10,000, pH 7.5) the living cytoplasm and the nucleus show green fluorescence. Acridinorange is absorbed electrostatically by the albuminoid components of the living protoplasm. But, in the living protoplasm, absorption is limited so that even after being stained with much excess dye, the protoplasm absorbs the acridinorange in low concentration only. Consequently the result is a green fluorescence of the living protoplasm. If, however, dead cells are stained with the same solution of acridinorange the dead protoplasm will develop a bright copper-colored fluorescence after a short time. The albuminoid components of dead protoplasm are able to absorb the acridinorange cations in such high concentration that a strong accumulation of the acridinorange cations, caused by electroabsorption, will result. Consequently the dead protoplasm shows a red fluorescence.

By a consideration of the position of the isoelectrical points of the different parts of the cell, the acridinorange method may be elaborated to a technique that makes possible an exact distinction between living and dead protoplasm. Strugger (4) and Strugger and Hilbrich (5) elaborated this method for the vital staining of microorganisms and for the distinction, by fluorescence microscope examination, of living and dead bacterial cells. It has been

* 

Acridinorange
Chloride salt of 3,6-tetramethyldiaminoacridin
Manufactured by I. G. Farbenindustrie, standardized Dye Bayer.

** *An analysis lamp manufactured by Quarzlampengesellschaft Hanau.*

shown that the protoplasm of yeast cells and all forms of bacteria can be vitally stained with acridinorange. This vital staining is so harmless that culture and animal experiments can be performed, with optimum results, with bacteria so stained. As the vital staining of bacteria with acridinorange allows a very clear observation of the cell walls and protoplasm, this method by which the stain is so simply and easily applied, is superior from every point of view to the methods of staining smears used in bacteriology until now.

The Method of Staining Suspensions of Soil Bacteria with Acridinorange

For the fluorescence microscope observation of the autochthonic bacteria of soil, I thought it was theoretically important to make tests with acridinorange. The first provisional tests gave excellent results.

When a suspension of soil is stained for 10 min. with a 1 : 2000 solution of acridinorange, made with tap water, the soil absorbs most of the dissolved dye. The humus ingredients have an electronegative charge and are able to absorb the acridinorange cations very strongly. Therefore all humus particles and all particles of soil covered with humus shine in copper-colored fluorescence under the fluorescence microscope. The bacteria, however, living on the particles and layers of humus, allow an intravital absorption of acridinorange in small concentration only, and therefore they have a bright green fluorescence. Thus, it is possible to observe the green fluorescent living bacteria in excellent contrast against the red background of the stained soil.

The staining of the soil was carried out as follows. In each of five test tubes was placed 1 gm. of sifted soil. To each tube was then added 10 cc. of acridinorange solution, made with tap or spring water, and each was strongly shaken. As the different kinds of soil may differ in their ability to absorb the stain, the same kind of soil must be stained with five different concentrations of acridinorange (1 : 1000, 1 : 2000, 1 : 3000, 1 : 4000, 1 : 5000). In this way the concentration of acridinorange most suitable for the staining of the soil may be ascertained empirically. If the concentration is too low, the acridinorange solution will be totally discolored within a few minutes after being shaken, and the humus ingredients of the soil are not stained red enough. If the concentration is too high for the soil concerned, too much excess stain remains in the solution, hindering observation. When only a little excess stain remains in the solution after heavy shaking, the concentration is satisfactory. Consequently the most suitable acridinorange concentration must depend on the proportion of humus in the soil. Provided that the humus particles are fully saturated with acridinorange, the fluorescence microscope observation of the bacteria living on the soil is possible.

The staining of the soil is completed very quickly. Generally a few minutes is sufficient but it is advisable to wait 5 or 10 min. before making the fluorescence microscope observation.

Two methods may be used for the preparation of the slides.

1. For the qualitative analysis of the autochthonic bacteria of the soil the stained soil suspension must first be strongly centrifuged. The solution remaining on top is decanted and a small quantity of the soil sediment is well mixed in a drop of paraffin oil on a slide. Thus many soil particles are introduced into the paraffin and, after covering the mount with a coverslip, a most favorable observation with the fluorescence microscope is possible.

2. For counts a small drop is taken with a needle, from the stained and shaken suspension of soil, put on a slide, and covered with a coverslip. Usually the small amount of excess dye present does not interfere with the observation of the bacteria under the fluorescence microscope.

The examination of these preparations has to be carried out with a luminous blue light fluorescence microscope.* As the bacteria are extremely small, an oil immersion lens and a high magnification have to be used. As immersion fluid the scarcely fluorescent paraffin oil is very suitable.

In the preparation of slides for counts, a counter of 20μ depth combined with a counting ocular is used. Taking into account the volume of the stained solution and the weight of the soil, a quantitative analysis of the results is possible.

If a preparation is made correctly the particles of soil covered with humus and the particles of humus shine in dim red fluorescence and the bacteria living on the particles of soil are green. When the bacteria of soil are killed by certain treatments before staining, no green colored bacteria are to be seen on the red fluorescent particles of soil.

The vitally stained bacteria of soil can be used for further cultivation. For this purpose, the smallest particles of soil are isolated and grown in a microculture in a drop of nutritive solution under microscopical control. Within a day, numerous bacterial cells develop from the cells stained green. They differ considerably in size and form from the autochthonic bacteria. By these experiments definite proof can be given that the small rod-like and sphere-shaped forms, shining green after being stained with acridinorange, are identical, indeed, with the autochthonic bacteria of the soil.

Results

Most of the bacteria of soil live on the humus layers of the soil particles. The cells are either isolated or united in zoogloea-like colonies. Only a few live free in the water. The autochthonic bacteria are so small that they can be observed only with the best objectives. Their size is between 0.3 and 1μ. The forms found by cultivation are far larger and cannot be identified at once with the autochthonic forms.

** The blue light fluorescence microscope is obtained by inserting blue filters (BG1, 2 mm. thick and BG3, 1¼ mm. thick, manufactured by Schott and Gen. Jena, and 5% copper sulphate in a cuvette 2 to 3 cm. thick, or a solution of concentrated cuprammonium sulphate—$[Cu(NH_3)_4]SO_4$—in a cuvette 2 to 5 cm. thick) in a luminous fluorescence microscope and by replacing the Euphos stop filter by an OG1 filter, 1 mm. thick, from Schott and Gen. Jena. Glass condensing lenses have been used.*

By numerous counts it was ascertained that, depending on the quality of the soil, 1 gm. of soil contains 500 to 10,000 million bacteria. These figures refer to the living bacteria only. Only under unfavorable conditions were many dead bacteria found. After dryness, especially, the percentage of dead cells was found to be higher than usual.

TABLE I

Kind of soil	Depth of counter, mm.	Water content per gm. of fresh soil, %	Average no. of bacteria per field of view*	Rounded no. of bacteria per gm. of soil (fresh wt.)	Rounded no. of bacteria per gm. of soil (dry wt.)	Statistic figure for no. of zoogloea per 10 fields of view**
Garden soil, surface test	0.02	—	11.2	3,900,000,000	—	—
Garden soil, surface test	0.02	13.8	13.6	4,860,000,000	5,640,000,000	—
Garden soil, surface test	0.05	12.9	24.6	3,786,000,000	4,347,000,000	1.6
Same soil as above, dried 24 hr., at 37° C.	0.05	1.5	14.13	1,982,000,000	2,012,000,000	0.33
Same soil as above, watered 24 hr.	0.05	32.2	21.6	3,099,000,000	4,571,000,000	0.9
Best meadow soil, surface test	0.05	30.0	42.4	6,048,000,000	8,640,000,000	4.5
Sandy soil of heath, surface test	0.05	0.5	8.94	981,000,000	1,038,000,000	—

** The counts have been performed with an apochromat objective n.A. 1.3, 120× and a counting ocular 10×.*

*** The number of zoogloea observed in 10 fields of view is noted and after counting several preparations the average value is taken.*

In Table I are given some results of counts of bacterial numbers. It is interesting to note the very marked effect of dryness on the life of soil bacteria. It may be seen, for example, that dryness lasting for only 24 hr. can markedly reduce the number of living bacteria in garden soil.

Conclusion

The observations reported in this paper show that it is possible to carry out a practically useful, rapid estimation of the number of bacteria in soil by staining the soil with acridinorange and examining it under the fluorescence microscope. The number of bacteria in the soil changes at a very rapid rate. Generally the number rises with an increase in the quantity of humus. The distribution of the bacteria in the soil profile, too, has been investigated by this method. Generally it has been confirmed that the quantity of bacteria is at its maximum within the rhizosphere, while in the B-horizon, where fewer plant roots are growing, the quantity very rapidly decreases.

Using this method of observation described, it is now possible to investigate directly the influence of various methods of cultivation or manuring as well as the influence of climatic factors on the bacteria of soil. The acridinorange method is most suitable for the study of the rhizosphere.

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A CONTRIBUTION TO THE STUDY OF DORMANCY IN SEEDS

LACTUCA SATIVA L.[1]

By C. W. Leggatt[2]

Abstract

The ease with which lettuce seeds may be made dormant by placing them under germinating conditions in blue light suggested that this kind of seed would provide satisfactory material for a study of certain aspects of dormancy in seeds. Studies have been made of the behavior of lettuce seeds under varied conditions of moisture, atmosphere, color of light, and integrity of the testa. Measurements of the absolute respiration have provided curves of the drift with time of carbon dioxide output, oxygen uptake, and of respiratory quotient. Experiments with substances that might induce dormancy and light-sensitiveness similar to that induced by blue light have been made. The bearing of the results obtained upon possible metabolic changes in the seed are discussed.

The possibility that the observed effects of light upon seed germination are due to changes in the permeability of the seed coat induced by light is considered as untenable, but this does not exclude the fact that the seed coat is a restrictive agency to the free passage of oxygen, carbon dioxide, and other substances. Indeed, in any description of the probable metabolic state, this must certainly be considered. While it has not been possible to formulate a definite biochemical scheme to account for blue light effects, the experimental evidence suggests that 'carbon dioxide zymasis' may be promoted by blue light and that carbon dioxide may be one of the inhibiting factors. The blue light dormant condition is characterized by depressed respiration but without more disturbance of the equilibrium of the pre- and postglycolytic phases of respiratory metabolism than is found to be characteristic of uninhibited seeds as reported by other workers.

The results of this research suggest that further elucidation of the phenomenon of dormancy should be sought in the direction of more extended studies of the respiration of dormant and non-dormant seeds.

Introduction

The failure of seeds to germinate promptly when provided with conditions 'ordinarily' suitable for the germination of seed of the species is a phenomenon of rather frequent occurrence. The existence and degree of dormancy are generally correlated inversely with the time elapsed since harvest; after a period of maturation, the dormancy becomes broken and the conditions of moisture, aeration, temperature, and substrate under which the seeds will then germinate may be considered as those 'ordinarily' suitable for seeds of the species.

Dormancy is a problem of great practical importance in the arts of agriculture, horticulture, and sylviculture, and both on this account and on that of its intrinsic interest it has received the attention of a large number of workers. It presents itself as a problem in two ways: failure of prompt

[1] *Manuscript received December 3, 1947.*

Contribution from the Department of Botany, University of Toronto, Toronto, Ont. An abridgment of a thesis presented in May, 1936, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] *In charge of Seed Research, Seed Research Laboratory, Division of Plant Products, Department of Agriculture, Ottawa.*

germination, under favorable conditions, of sound, healthy seeds may cause serious loss to the grower while, to the seed analyst, responsible for the enforcement of the law or for the issuing of reports that form the basis of sale, it is often a source of great embarrassment and may result in financial loss to the trader. From the point of view of physiology and ecology, germination and dormancy in seeds appear to form part of a group of related phenomena having to do with the processes of growth that are of the highest interest and importance.

Dormancy in seeds presents a very wide field and accordingly, in the present paper it has been necessary to confine the study to one, somewhat restricted, phase. Nevertheless, it is possible that the various kinds of dormancy that have been described may be more closely connected than some have thought, so that observations, even in a restricted phase may have a fairly wide application.

I. Experiments with *Lactuca sativa* L. Preliminary Tests

In the preliminary experiments, confirmation was soon obtained of the inhibiting effect of blue light and the promotive effect of red light on lettuce seed germination as reported by Flint (2) and Flint and McAlister (3). Owing to lack of time with regard to the number of tests that had to be made, it was unfortunately necessary in this preliminary work to use rather small samples and, since the seed was rapidly undergoing a drift in metabolic equilibrium at the time, it was not possible to repeat experiments exactly. This drift is shown in Fig. 1 in which germination in the dark after between two and three days is plotted against the date when the test was made. It is evident that the seed was dark-sensitive up to the middle of February, after which time light was not necessary for its complete germination. The same sample on Jan. 15, 1935, germinated 95% after $2\frac{3}{4}$ days with exposure to daylight. It was a sample of the variety Early Curled Simpson and was used throughout these preliminary tests.

The dark tests were conducted by setting the seeds on damp blotters in covered Petri dishes, wrapped in impenetrable black paper and set in a dark germinator at 20° C. Originally, dark Petri dishes had been prepared by covering them outside and inside with lead foil by means of Duco Household Cement and, when thoroughly dry, painting them all over with two coats of flat black. However, it was found that as long as the smell of the Duco cement persisted, seeds were completely inhibited from germinating. This was proved by smearing a little of the cement on the insides of undarkened dishes, which were used for tests when the cement had hardened thoroughly; such tests showed complete inhibition until the smell had gone.

The foregoing has a bearing on the following experiment (No. 4). Two hundred seeds were set to germinate at 20° C. on damp blotting paper in each of the following:—darkened Petri dishes (as above), Petri dishes wrapped in black paper, and in Petri dishes wrapped in blue cellophane.

Of the seeds remaining ungerminated after two days, 50 from each test were cut in such a way that the pericarp only was ruptured and 50 in such a way that the testa also was ruptured, the seeds having been allowed, previous

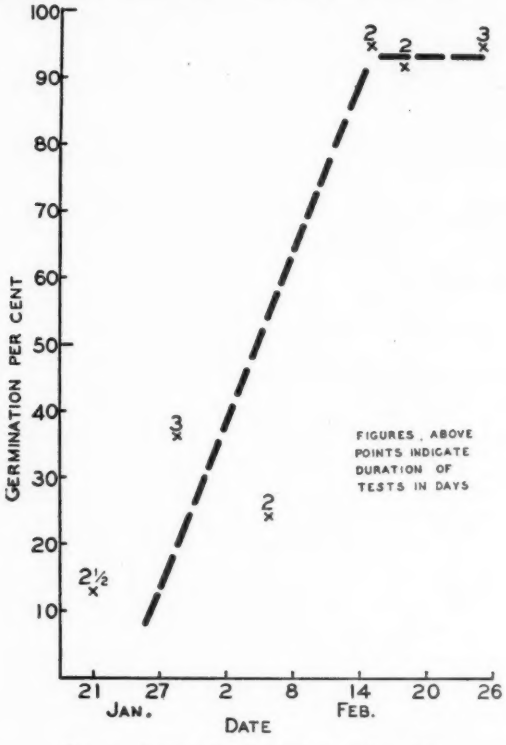

FIG. 1. *Germination in dark at various dates.*

to this treatment, to dry in the dark at room temperature. The seeds so treated were then set to germinate in the darkened dishes in which Duco had been used, the effects of the Duco not having been realized at that time. (Henceforward these are called the Duco dark dishes.) The germination results are given in Table I.

Four points of especial significance are to be noted:—

(1). Where the pericarp only was ruptured, very low germination followed.

(2). Where the testa also was ruptured, germination is practically complete in all tests after 14 days.

(3). The 'Duco dormant' seeds were the quickest to recover while the 'dark dormant' and the 'blue light' dormant seeds lagged behind, the latter most of all.

TABLE I

EFFECT OF RUPTURE OF PERICARP AND OF TESTA ON DORMANT SEEDS

Pretreatment	Germ., % two days	Clipped	Germination % after: Days						
			2	4	6	8½	10¾	14	16
'Duco dark'	0	Pericarp	0	0	0	0	2	4	4
		Testa	72	92	96	Remainder dead			
Black paper dark	24.5	Pericarp	6	6	6	6	8	10	10
		Testa	18	52	62	70	72	90	90
Blue light	0.5	Pericarp	0	0	0	0	0	Thrown out in error	
		Testa	12	34	50	60	70	92	92

NOTE: *During each count the seeds were subjected to a brief exposure to light.*

(4). The Duco dark dishes did not produce inhibition when the testa was ruptured.

It was found later that slight differences in moisture had an appreciable effect on germination, so that no significance can be definitely assigned to differences between the 'black paper dark' and 'blue light' lots. However, it is believed that this source of error in the present case is very slight since Petri dishes of the same size were used, the blotters were uniform, cut to the same size, and dipped and drained for the same time when the seeds were planted.

Expt. No. 6 was in part a repetition of Expt. 4, but Duco dark dishes were not used and the intact seeds (neither testa nor pericarp ruptured) were set to germinate in light, while the seeds with testa ruptured were set to germinate in blotters, wrapped in black paper and placed in flat tins. The date of this experiment was Feb. 16, 1935, at which time the seed had advanced rather far in its metabolic drift. The intact seeds were set to germinate, half in 'clear' light, i.e. with the Petri dishes wrapped in clear cellophane, and half in red light, the dishes being wrapped in red cellophane. Two hundred seeds were originally set in the dark and in blue light and of the ungerminated seeds there were sufficient for 30 each in red and clear light from the dark lot and 50 each in red and clear from the blue light lot, while in both lots there were 50 seeds with testa clipped. The results are presented in Table II.

In this experiment the clipped seeds have again shown much faster germination than the unclipped, but complete germination has been secured in 10 days, of intact seeds in light, as compared with their very low germination in the dark in Expt. 4. In both cases the 'clear' light has promoted germination

TABLE II

DORMANT SEEDS INTACT AND WITH CLIPPED TESTA

Pretreatment	Germ., % two days	Subsequent treatment		Germination % after: Days 2	4	6	8	10
Dark Lead foil and black paper	43.5	Intact	Red	0	3	20	47	93
			Clear	0	13	90*	90	90
		Clipped, dark		98		Thrown out		
Blue light	0.5	Intact	Red	0	10	50	86	98
			Clear	0	22	78	94	94
		Clipped, dark		98		Thrown out		

* *This test was a little moister than the others, although all appeared to have ample moisture for germination under normal conditions.*

somewhat faster than red light. This contradicts a previous observation (Expt. 2) in which the reverse was the case, but it is probable that the metabolic drift already referred to may have changed the response of the seeds in this respect. Further, the intensity of the light was considerably higher in the 'clear' than in the red light tests and this would also contribute to the difference.

During the course of the above work, certain observations had brought up the question as to whether the time of day when the tests were set had any influence upon the results. The source of light had been daylight with its natural fluctuations. Accordingly Expt. 9 was carried out to test this. Eight lots of 100 seeds were planted on uniform blotters uniformly moistened in covered Petri dishes and wrapped in blue cellophane. Four lots were planted at 9 p.m. and four lots at 9. a.m. Table III presents the results:—

TABLE III

EFFECT OF DAY PRECEDING NIGHT AND NIGHT DAY

Test	Planted	Period	Germination	Significance*
a	p.m.	3 N 2 D	1	$\chi^2 = 19.606$
b	p.m.	3 N 3 D	1	
c	p.m.	4 N 3 D	2	$n = 1$
d	p.m.	4 N 4 D	1	
e	a.m.	3 D 2 N	12	$P < .01$
f	a.m.	3 D 3 N	4	
g	a.m.	4 D 3 N	9	
h	a.m.	4 D 4 N	6	

* *Fisher, R. A. Statistical methods for research workers, 3rd ed. p. 70.*

From these results it appears that the total period before the counts were made has no bearing on the germination results but that significantly higher results were secured when day preceded night than in the reverse case. All sprouts were well grown so that the seeds either grew uninhibited, having started early, or were completely inhibited. The test of significance was based on the assumption that the N–D tests could be taken as a group having produced five sprouts out of 400 seeds and the D–N tests as another group with 31 sprouts out of 400 seeds. The value of P is so small that the two results cannot be considered as random deviates of the same population.

Working on the assumption that the blue light effects were due to an inhibitor soluble in water and capable of diffusion away if sufficient water was present, Expt. 11 was carried out. In this experiment 100 seeds were set in blue light using the same technique as in the preceding except that excess water was added so that the seeds were almost floating. In this experiment 15 sprouts were obtained in two days. During counting the seeds were exposed for about 30 sec. to diffuse daylight before being rewrapped in the blue cellophane. Two days later a total of 26 sprouts had been obtained and no further germination was secured. Since this test was planted at night and was thus an N–D test, for statistical purposes we may compare it with the N–D tests of Expt. 9. Here again $P < .01$ and the difference is to be considered as significant.

Expt. 12 was designed to test further the influence of moisture. No exact control of moisture was attempted, but with the dark gray blotter used, which becomes very dark on being thoroughly wetted, it was an easy matter to arrange the blotters in order of increasing depth of shade corresponding to increasing amounts of moisture. Initially three degrees were used, slight moisture, medium, and wet. The first was secured by quickly plunging the blotter into water and withdrawing it immediately, medium was with the blotter fully soaked, and wet was with sufficient additional moisture added to make the surface appear quite wet, with free water at the edges. At the end of the experiment it was found that slight drying had occurred, and that somewhat unevenly, so that it was possible to differentiate five degrees of moisture. In each of the three initial degrees of moisture, 50 seeds were set in dark and 50 in red light. The results follow in Table IV. Expt. 11 is also included.

These results clearly indicate that excess moisture is needed to secure complete germination, but that degrees of moisture that are limiting in dark are not so in red light. Germination remains incomplete even in free water in blue light.

Several points of special significance emerge from these preliminary tests. It is evident that, with the rupture of the testa, the agent conditioning dormancy is rendered inactive, while a similar effect is brought about by the presence of excess moisture, and that most readily in red light, least readily in blue light, and to an intermediate degree in the dark.

TABLE IV

INFLUENCE OF THE DEGREE OF MOISTURE

Degree of moisture	Germination, %, in two days in:—		
	Red light	Dark	Blue light
1		8 (S)	
2	74 (S)		
3		14 (M)	
4	98 (M)		
5	98 (W)	100 (W)	(W) 15 (26 in four days)

Initial moisture: (S) = slight; (M) = medium, and (W) = wet.

These observations are all compatible with the postulation of an inhibitor that may escape on rupture of the testa or by leaching when excess water is present, and that is present to the greatest degree in blue light, and to the least degree in red light.

The experiments in which seeds set to germinate in blue light were subjected either to light first or to darkness first demonstrate that there is a critical time before which blue light experience is not effective. That complete germination was not secured in those cases where light preceded darkness is to be ascribed to the probability that the critical period had been passed by the majority of the seeds before the initial light experience ended.

The results so far secured led to a tentative hypothesis that the effect of light was to render the semipermeable seed coat permeable, permitting the escape of an inhibitor that normally developed during the initial stages of germination, red light being specially active in this regard and blue light, not only inactive, but, if anything, tending to render the seed coat somewhat less permeable. Carbon dioxide suggested itself as a possible inhibitor; if it were, seeds set to germinate in an atmosphere of pure carbon dioxide would be expected to become dormant. In the next two sections these suggestions were explored.

II. Further Experiments on Germination of Lettuce Seeds

Expt. N.S. VII

In the respiratory flasks described in Section V, two grams of seed were set at 20° C., with humidified circulating air in blue light. The light failed at an unknown time but evidently had been adequate since no germination occurred up to 89 hr. After this treatment the seeds were dried at room temperature in the light and two lots of 100 seeds each were pricked and set to germinate at 20° C. in clear light; a further 200 seeds, unpricked, were set to germinate immediately.

Expt. N.S. VIIa

A similar experiment was carried out, but in clear light, and the respiratory chamber was filled with carbon dioxide and sealed. After 94 hr. continuous

light, germination was found to have been completely inhibited. The seeds were then treated exactly as in N.S. VII.

The results of Expts. N.S. VII and N.S. VII*a* are shown in the graphs, Fig. 2.

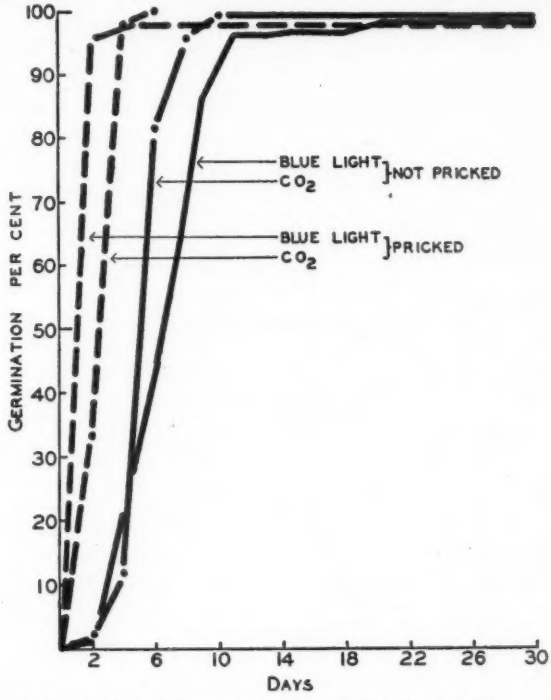

FIG. 2. *Experiments N.S. VII and N.S. VIIa—germination in light of seeds pretreated with blue light and carbon dioxide.*

Expt. N.S. XIV

This experiment was practically a repetition of Expt. N.S. VII, except that 1 gm. of seed was set in duplicate at 20° C., for 96 hr. in continuous blue light. From each lot, 2 × 75 seeds pricked and 2 × 75 unpricked were set to germinate in the dark at room temperature (close to 20° C.), and the same number pricked and unpricked were set to germinate in daylight. The corresponding replicates all gave very concordant results, the averages being shown in the graphs, Fig. 3. Results are shown up to 91 days. Ungerminated seeds still appeared fresh after this time.

III. Experiments to Determine Changes in Fats

Since the layer responsible for the semipermeability of the testa of lettuce seed is lipoid in character, any increase in permeability would be expected to be due to lypolysis, a process that could be detected by increase in free acid.

For this purpose a series of experiments was carried out to determine, by the methods commonly employed to determine saponification and acid numbers, and ester values, the amounts of free acid, total fatty acid and fats, all expressed as oleic acid radical per gram of seed, in dry seeds and in seeds after being exposed to germination conditions in blue light and in clear light. It was found, however, that the amount of fats in lettuce seeds is so high (36.6 to 39.9% according to my analyses) that changes in the seed coats evidently could not be traced by this method.

Expt. N.S. XVIII

In a further attempt to trace such changes, seeds were sectioned dry and also after imbibition in clear light and in blue light. The conspicuous semipermeable membrane of the inner epidermis of the integument described by Borthwick and Robbins (1), gave the characteristic orange color of suberin

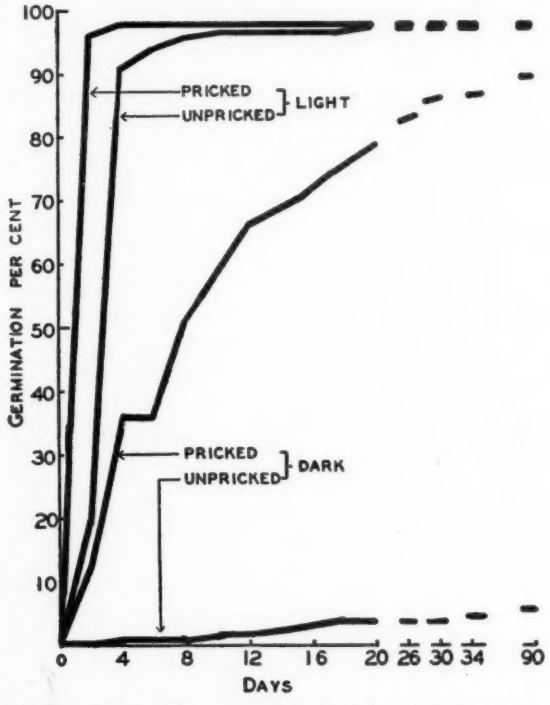

FIG. 3. *Experiment N.S. XIV—germination in light and darkness of seeds pretreated with blue light.*

with Sudan III. With Nile blue sulphate, after differentiation with 2% acetic acid, neither the characteristic pink of fats nor the blue of free fatty acids could be detected in any of the preparations.

IV. Discussion of Expts. N.S. VII, N.S. VII*a*, and N.S. XIV

Results of Expts. N.S. VII, N.S. VII*a*, and N.S. XIV have shown:—

(1*a*). That after seeds have become blue light dormant, rupturing the testa by clipping or pricking permits germination to proceed.

(1*b*). That after 24 hr. by blue light treatment followed by drying, however, unpricked seeds germinated almost as fast as pricked seeds in clear light, whereas in the dark, even pricked seeds showed marked retardation, while unpricked seeds in the dark germinated only to a very low percentage. It should be remarked here that the blue light pretreatment of seeds in N.S. XIV was much more intense than in Expt. 6, which may account for the slower germination of pricked seeds in the dark in the former than in the latter.

It seems a legitimate conclusion from this that the effect of blue light is not primarily upon the seed coat, since if it were, we should have expected little difference between germination in light and in darkness once the testa had been ruptured by pricking. The fact that adequate seed ventilation is provided by rupturing the testa is indicated by the prompt germination of clipped seeds in Duco dishes in which unclipped seeds were inhibited from germinating, as was shown in the preliminary section. This conclusion is further supported by the negative result of Expt. N.S. XVIII.

(2). Carbon dioxide, at a concentration of 100%, inhibits germination.

Germination curves for pricked and unpricked seeds in light resemble similar curves for blue light – treated seeds far more closely than the latter resemble those of blue light – treated seeds in the dark. (Compare graphs for Expts. N.S. VII and N.S. XIV, Figs. 2 and 3.)

Thus, while the effect of carbon dioxide is not identical, it is at least similar to that of blue light—probably a difference of degree rather than kind.

V. Respiratory Studies with *Lactuca sativa* L.

Material

Respiratory studies were carried out on a particularly good sample of Hansen lettuce of high germination (99 to 100%), with large plump seeds and of the 1934 crop, thus past that period in its metabolic drift that would make light a necessary requirement for satisfactory germination.

Methods

The Respiratory Chamber

Exact details of the respiratory chambers need not be given. Erlenmeyer flasks (250 cc.) were used and so arranged that samples of the respired gases could be withdrawn without change of pressure and the whole chamber could be flushed out with fresh, humidified air when needed. The volumes of the chambers were determined to an accuracy of 0.5%, allowance being made for seeds, filter paper, and added water.

At the bottom of each flask was placed a circular filter paper, the proper amount of water added, and the seeds sprinkled in an even layer over it.

Measurement of Respiration

The respired gases were analyzed by means of a Haldane respirometer using a gas burette graduated in 1 cc. units up to 7 cc. thereafter in 0.01 cc. units up to 10 cc. Readings could be made by estimating to 0.001 cc. Temperature fluctuations were automatically compensated by a dummy burette, the two burettes being enclosed in a water jacket. Barometric fluctuations were negligible during the course of a determination.

Procedure

In all cases exactly 2 gm. of seed was used. The seeds having been spread as described on the wetted and drained filter, a measured volume of water was added (6 cc. in the first experiments, 3 cc. subsequently), and the flasks were then flushed for five minutes. The air used for flushing, taken either from outside or from the compressed-air lines, was analyzed at frequent intervals, these analyses being used as blanks.

The results of analysis were calculated to cubic centimeters per gram per hour. Duplicate analyses were made in all cases and where reasonably close agreement was not secured, additional analyses were made. In addition, each entire experiment was done in duplicate although, as will be seen, it was not possible always to duplicate an experiment exactly.

The respiratory chambers were wrapped in red, blue, or clear cellophane before being placed in constant temperature tanks for the period of respiration. The light source was a 250 w. Mazda lamp placed in a glass cylinder immersed in the tank, and spaced about 6 in. from the respiratory chambers.

Tests with red and clear light could only be carried out during a limited time, since the seeds germinated and thus the respiration ceased to be characteristic of seeds. Blue light tests, however, were prolonged since germination was completely inhibited.

The work was done during September, October, and November, 1935.

Results

Expt. R I

Seeds allowed to imbibe 14 hr. with constant illumination in sealed chamber and respired air analyzed. The following results were secured, expressed as percentages and in cubic centimeters per gram per hour. The temperature in the constant temperature tank was 20° C.

Color of light	Replicate analysis	CO_2		O_2		R. Q.
		%	Cc. per gm./hr.	%	Cc. per gm./hr.	
Blue	1	2.84	0.2121	3.60	0.2697	0.787
	2	2.87		3.66		
Clear	1	3.67	0.2788	4.46	0.3412	0.817
	2	3.66		4.51		

Expt. R Ia

This was a duplicate of the above except that the period of respiration was 16½ hr. The temperature control failed during the respiratory period and at the end the temperature was 23° C., having started at 20° C. The results follow:

Color of light	Replicate analysis	CO_2		O_2		R. Q.
		%	Cc. per gm./hr.	%	Cc. per gm./hr.	
Blue	1	3.64	0.2353	4.36	0.2501	0.839
	2	3.65		4.33		
Clear	1	5.53	0.3485	6.37	0.4024	0.866
	2	5.53		6.40		

Expt. R II

This was a further replicate of Expt. R I as far as the blue light was concerned, but instead of clear, red cellophane was used. The period of respiration was 14 hr. and the temperature, while the control was still somewhat uncertain, was between 20° C. and 21° C.

Color of light	Replicate analysis	CO_2		O_2		R. Q.
		%	Cc. per gm./hr.	%	Cc. per gm./hr.	
Blue	1	2.95	0.2244	3.71	0.2840	0.790
	2	2.95		3.76		
Red	1	3.93	0.2914	4.83	0.3592	0.810
	2	3.92		4.84		

Expt. R III and Expt. R IIIa

In these, blue and red were again the colors used but the respiratory period was reduced to four hours. Owing to several mishaps only one analysis has been accepted from Expt. R III since it checks with the analysis in Expt. III*a*. Temperature control was satisfactory at 20.25° C. The one test in R III was with blue light. The following are the results of these two experiments:—

Color of light	Experiment and replicate	CO_2		O_2		R. Q.
		%	Cc. per gm./hr.	%	Cc. per gm./hr.	
Blue	R III 1	0.86	0.2437	0.98	0.2727	0.895
	R III*a* 1	0.91		1.00		
Red	R III*a* 1	0.97	0.2636	1.04	0.2851	0.920
	2	0.99		1.08		

Expt. R IV

This was similar to the previous experiments with a respiratory period of four hours but with red and clear cellophane. Temperature control was satisfactory at 20° C. to 20.25° C. The results follow:—

Color of light	Replicate analysis	CO_2		O_2		R. Q.
		%	Cc. per gm./hr.	%	Cc. per gm./hr.	
Red	1	1.01	0.2688	1.19	0.3168	0.85
	2	1.01		1.18		
Clear	1	1.10	0.2964	1.24	0.3224	0.89
	2	1.11		1.24		

Expt. R V

This experiment constituted in part a duplicate of RI and in addition the blue light test was continued for 95½ hr., being sampled at intervals. In this experiment the respiratory flasks were flushed with air after each sampling and remained sealed until the next sample was drawn. Thus the respiratory periods sampled vary considerably. The temperature was 20.75° C. at the start and 20.5° C. at the end. Results:

Color of light	Analysis	Respiratory period, hr.	Total time elapsed	CO_2		O_2		R. Q.
				%	Cc. per gm./hr.	%	Cc. per gm./hr.	
Clear		3.5	3.5	0.94	0.2833	0.985	0.2969	0.95
Blue	*a*	3.5	3.5	0.91	0.2808	0.98	0.3024	0.93
"	*b*	3.5	7.1	1.135	0.3502	1.32	0.4073	0.86
"	*c*	16.2	23.3	3.335	0.2227	4.16	0.2778	0.80
"	*d*	3.5	27.0	0.85	0.2623	1.03	0.3024	0.83
"	*e*	44.5	71.5	6.32	0.1535	7.91	0.1906	0.80
"	*f*	3.5	75.1	1.16	0.3579	1.16	0.3579	1.00
"	*g*	3.5	78.7	0.93	0.2870	1.09	0.3363	0.85
"	*h*	16.7	95.5	3.71	0.2401	4.59	0.2974	0.81

Expt. R VI

Owing to the strongly depressive effects observed due to increasing accumulation of carbon dioxide and diminution of oxygen in the long respiratory periods in the preceding experiment, another experiment was carried out in blue light similar to R V except that the flasks were continually flushed with humidified air until sealed for the respiratory period. The temperature varied between 20° C. and 20.25° C. during the experiment. Results:—

Analysis	Respiratory period, hr.	Total time elapsed	CO_2		O_2		R. Q.
			%	Cc. per gm./hr.	%	Cc. per gm./hr.	
a	2.8	19	0.665	0.2476	0.80	0.2979	0.83
b	3	22.2	0.585	0.2057	0.735	0.2602	0.79
c	3	43	0.38	0.1336	0.71	0.2497	0.54
d	3	46.2	0.44	0.1547	0.73	0.2567	0.60
e	3	49.4	0.44	0.1547	0.69	0.2427	0.64
f	3	52.6	0.49	0.1723	0.635	0.2233	0.77
g	3	67.1	0.395	0.1389	0.67	0.2356	0.59
h	2.75	114.8	0.57	0.2187	0.96	0.3683	0.59

Note: During entire respiratory period, seeds held in blue light.

Expt. R VIa

This was a repetition of the previous experiment. Results are tabulated below:—

Analysis	Respiratory period, hr.	Total time elapsed	CO_2		O_2		R. Q.
			%	Cc. per gm./hr.	%	Cc. per gm./hr.	
a	3	19	0.47	0.1653	0.75	0.2637	0.63
b	3	22.1	0.65	0.2286	1.04	0.3657	0.63
c	3	43	0.415	0.1459	0.67	0.2356	0.62
d	3	46.1	0.57	0.2004	0.85	0.2989	0.67
e	3.6	49.9	0.73	0.2120	1.12	0.3252	0.66
f	2.8	52.8	0.73	0.2767	1.07	0.4056	0.69
g	2.75	66.8	0.325	0.1247	0.49	0.1880	0.66
h	3	90.8	0.665	0.2339	1.09	0.3922	0.61

Note: During entire respiratory period, seeds held in blue light.

In Table V, Expts. R V to R VI*a* are summarized in such a way as to show chronological relationship, the figures in bold face type being those that are used for plotting the graph, Fig. 4, and that have been selected for the following reasons. With regard to R V, it has already been mentioned that prolonged periods with the seeds in sealed vessels resulted in a marked depression of the respiratory rate. For this reason, only those results are accepted that represent data for short respiratory periods. Examination of the data for Expts. R VI and R VI*a* shows that in general lower values for oxygen, carbon dioxide, and respiratory quotients are obtained after long periods of ventilation than after short periods of ventilation such as those between respiratory periods succeeding one another at short intervals. The constant occurrence of this phenomenon suggests that a cumulative factor other than blue light is operating to give progressively higher values until a long ventilating period intervenes to alleviate the condition. For this reason only those values are accepted that represent data obtained after a long period of ventilation. Results secured after 70 hr. are not accepted since mold growth became evident after this time.

TABLE V

SUMMARY OF EXPERIMENTS R V, R VI, AND R VI*a*

		Hours											
		3.5	7.1	19	22.1	23.3	27	43	46.1	49.6	52.7	67.0	71.5
CO_2	R V	**.281**	**.350**			.223	**.262**						.154
	R VI			**.248**	.206			**.134**	.155	.155	.172	**.139**	
	R VI*a*			**.165**	.229			**.146**	.200	.212	.277	**.125**	
O_2	R V	**.302**	**.407**			.288	**.302**						.191
	R VI			**.298**	.260			**.250**	.257	.243	.223	**.236**	
	R VI*a*			**.264**	.366			**.236**	.299	.325	.406	**.188**	
R.Q.	R V	**.93**	**.86**			.80	**.83**						.80
	R VI			**.83**	.79			**.54**	.60	.64	.77	**.59**	
	R VI*a*			**.63**	.63			**.62**	.67	.66	.69	**.66**	

Examination of the graph, Fig. 4, reveals substantially a drift with time from higher initial values to lower final values both for carbon dioxide output, for oxygen uptake, and for respiratory quotient. The fact that the curves are, in general, at somewhat different levels may be ascribed to two causes; variations introduced by experimental and sampling errors and the fact that the experiments were separated by a considerable interval of time, Expt. R VI*a* having been carried out a month later than the other two; thus the samples may not have been at comparable stages in their metabolic drift. Bearing this in mind, the fact emerges that the final values reached at 70 hr. are of the same order of magnitude, and the shape of the curves suggests that the measures of respiration rate and of respiratory quotient are approaching values characteristic of blue light dormancy.

The curves given by R V may be regarded as representing initial stages of curves similar to those given by R VI and R VI*a*, and the differences between the curves as differences in the mode of progress to the same state exhibited by the several samples. These ideas may be schematized as in Fig. 5.

The work of Leach and Dent (4) on the respiration of fatty seeds has shown that the respiration rate of uninhibited seeds starts in the manner indicated by the upward slope of the curves shown in the above scheme, but that instead of the depression that begins at the seven hour point, the respiration continues to rise for a long time. This would indicate that the seven hour point represents a critical stage in the metabolic drift of lettuce seeds at which blue light begins to be operative, and is in striking agreement with the failure of blue light to produce complete inhibition when blue light experience is stopped before this stage is reached, as shown by the preliminary experiments reported in Section I. It should be mentioned that the seven hour point

is not an exactly determined one and that the true critical point may lie somewhat on one or the other side of it, or more probably over a more or less extended period owing to statistical differences in the responsiveness of different seeds to germinating conditions.

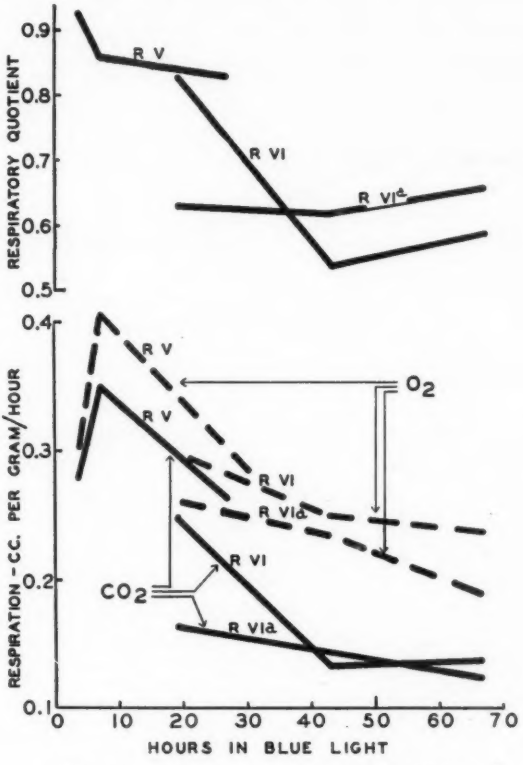

FIG. 4. *Respiration of lettuce seed in blue light.*

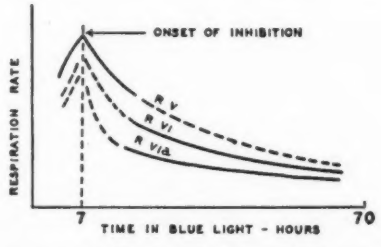

FIG. 5. *Modes of progress of different samples to respiration rates characteristic of blue light dormancy. Schematic.*

It is of interest to compare the drift of respiratory quotient in the case of inhibited lettuce seeds with that of uninhibited seeds as given by Leach and Dent. For this purpose we may use the results they give for seed of *Helianthus annuus*, belonging to the same botanical family as lettuce. In the graph, Fig. 6, are shown our own results for R V and R VI, which were done within a few days of each other, superposed upon theirs and reduced to the same percentage time scale. In both cases there is seen a fall to a minimum fol-

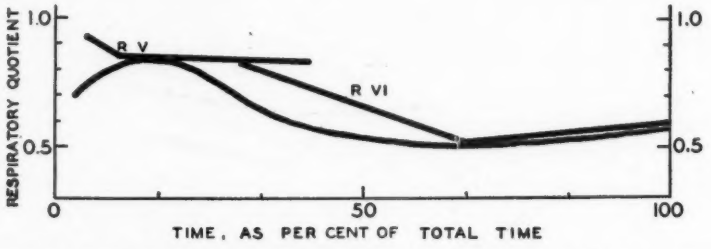

FIG. 6. *Drift of respiratory quotient for lettuce seeds compared with that for sunflower. Curve for sunflower from Leach and Dent.*

lowed by a gentle rise and also the absolute magnitudes are closely similar. The same gentle rise is also given by R VI*a*, but at a slightly higher level. For the time being, and in the absence of similar determinations on uninhibited lettuce seed, it is reasonable to suggest that the drifts of respiratory quotient in dormant and non-dormant lettuce seed are similar, while the curves of respiration deviate from one another sharply. This would point to an effectively equivalent depression of the pre- and postglycolytic phases as characteristic of blue light dormancy. It is evident that to substantiate this suggestion, more respiratory data are needed.*

The results of the experiments recorded up to the present point led to the exploration of an hypothesis based upon the effect of light upon the respiratory mechanism. This hypothesis postulated an anaerobic zymasis induced by blue light, but further consideration seems to indicate that anaerobic zymasis is improbable under the conditions existing within the seed, where there is no reason to suppose oxygen tensions below the extinction point exist. However, the hypothesis led to the experiments next to be recorded, and further discussion will be delayed until after these have been considered.

* *The question whether oxidative anabolism may play a part in the respiration of lettuce seeds and if so, whether the effect of blue light might change the fate of the products of OA (oxidative anabolism) was considered. It seems reasonable to suppose that the carbon that would be lost in the absence of OA, when anabolized may be incorporated as structural carbon. If the critical seven hour period should coincide with the initiation of growth processes with consequent demand for structural carbon, and if the products of OA are diverted from this goal by blue light, we might here find the explanation of the inhibition produced by blue light. In the absence of evidence that OA exists in the material in question we cannot develop this idea further at the present time, but suggest that it is a possibility worth following up.*

VI. Experiments to Induce Dormancy by Means Other than Blue Light

The hypothesis just mentioned suggested that the inhibiting substance was one of the products of anaerobic respiration.

This possibility was tested in the experiments that follow:

Expt. N.S. XIX

Two lots of 100 seeds were set in light and 2 × 100 in the dark, on top of filter papers in covered Petri dishes at 20° C. The filter papers were severally moistened with solutions of ethyl alcohol (1%), acetic acid (1%), and acetaldehyde (0.2%), and also glycerine 1% and 3%, using 2 × 100 seeds in each case, but in light only. A check test was set between blotters in the dark at 20° C., 2 × 100 seeds being used. Thus, in all, 18 × 100 seeds were used in this experiment. In moistening the filter papers, they were completely dipped in the solution until fully soaked and, after allowing the free water to drain off, were transferred to the Petri dishes. The seeds were then planted and 10 drops of solution added. The covered Petri dishes were then placed in humid germinators, the light tests being exposed to daylight, the dark tests being wrapped in sheets of wet, dark blotting paper and placed in dark chambers.

Results

Check tests.—Germination was practically complete (94.5%) in 46.5 hr.; 44.5 hr. later 2% more had germinated, and 23 hr. later another 0.5% germinated. The remaining 3% apparently went into dormancy, since no further germination was secured after 50 days, although the seeds still looked healthy.

Acetaldehyde (0.2%).—Germination was apparently somewhat stimulated at this concentration, since all good seeds germinated in 48 hr., a total of 98.5% in light and 99.5% in dark. Within the next two days, one weak sprout had developed in the light test and 23 days later another weak sprout had grown, while the remaining seed was evidently dead. The single ungerminated seed in the dark test was dead.

Acetic acid (1%).—The seeds were all killed and had swelled to twice their normal size.

Ethyl alcohol (1%).—This solution showed marked retardation of germination, both in light and dark. The latter gave somewhat conflicting results in the duplicates, so two more replicates of 100 seeds were run. These concorded well with each other and also with one of the other replicates, thus the three concordant tests were accepted.

Detailed results are shown on Fig. 7.

Glycerine (1% and 3%).—Germination was depressed by the 1% solution and almost completely inhibited by the 3% solution (2.5% germination) but the dried seeds showed no signs of dormancy.

Expt. N.S. XIXa

In this experiment 4 × 100 seeds were set (the same technique being followed), two in light and two in dark, using a solution of 2% ethyl alcohol. At this strength germination was almost completely inhibited as follows:—

Test	Seeds used	Sprouts after 49.5 hr.
Light 1	100	3
Light 2	100	0
Dark 1	100	1
Dark 2	100	0

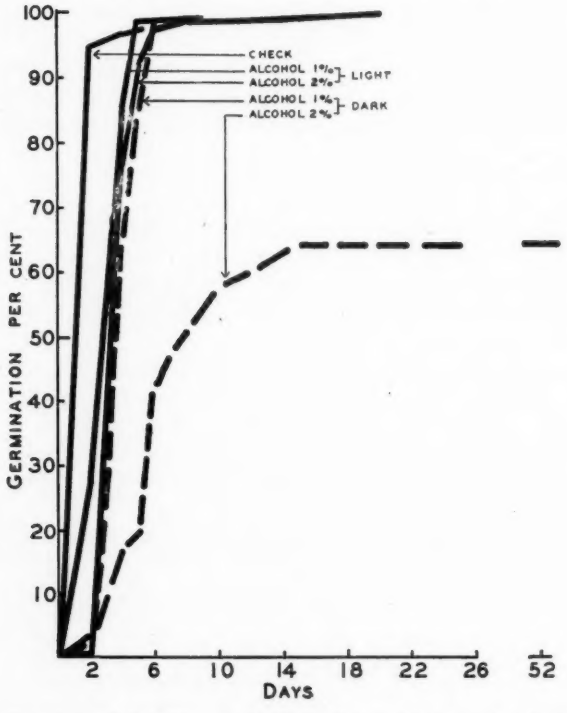

FIG. 7. *Experiment N.S. XIX—germination in light and darkness of seeds made dormant by alcohol.*

The ungerminated seeds were allowed to dry in the dark and were then replanted according to the following plan:

Original treatment	Subsequent treatment
(1) Light 1 (2) Dark 1	Dark, between blotters moistened in tap water, in dark cabinet, at 20° C.
(3) Light 2 (4) Dark 2	On top of filter papers under bell jars in Jacobsen apparatus, at 20° C., daylight.

Since the subsequent treatment evidently had much greater effect than the original treatment, and since (1) and (2) concorded reasonably well, and (3) and (4) very well, (1) and (2) have been averaged, as have (3) and (4) in the graph that is shown in Fig. 7.

Another experiment was also run using a concentration of 0.2% acetic acid. At this concentration the solution gave results almost identical with acetaldehyde, 0.2%; 98.25% germination was secured after 47 hr.

Expt. N.S. XIXb

This experiment was based upon the same general plan as the alcohol 2% test, but in this case 3% was used. At this strength inhibition of germination was complete. After the seeds had dried in the dark, 50 seeds of each replicate were pricked. Pricked and unpricked seeds were then replanted according to the following plan.

Original treatment	Subsequent treatment
(1) Light 1	P (pricked) Dark as in N.S. XIX*a*
(2) " 1	U (unpricked) " " " "
(3) " 2	P (pricked) Light " " "
(4) " 2	U (unpricked) " " " "
(5) Dark 1	P Dark " " "
(6) " 1	U " " " "
(7) " 2	P Light " " "
(8) " 2	U " " " "

Again, initial treatment seemed to have had little effect and excellent checks were given by (1) and (5), by (2) and (6), by (3) and (7), and by (4) and (8). These pairs have accordingly been averaged and the results are shown in Fig. 8.

Summary of Experiments N.S. XIX, XIXa, and XIXb and N.S. XIV

Expt. N.S. XIV showed that seeds treated with blue light under conditions otherwise suitable for germination, dried and then pricked, germinated as promptly in light as did the check test (Figs. 2 and 3). Similar seeds germinated in the dark showed marked retardation, but germination had reached the 90% point after 90 days. Unpricked seeds had germinated fairly promptly and completely in the light, but in the dark showed extreme retardation.

Expts. N.S. XIX, XIX*a*, and XIX*b* show that alcohol not only inhibits germination, but that the treated seeds have become dormant, even after drying. It is significant that this dormancy is coupled with light sensitiveness of a type similar to, though less marked than, that induced by blue light.

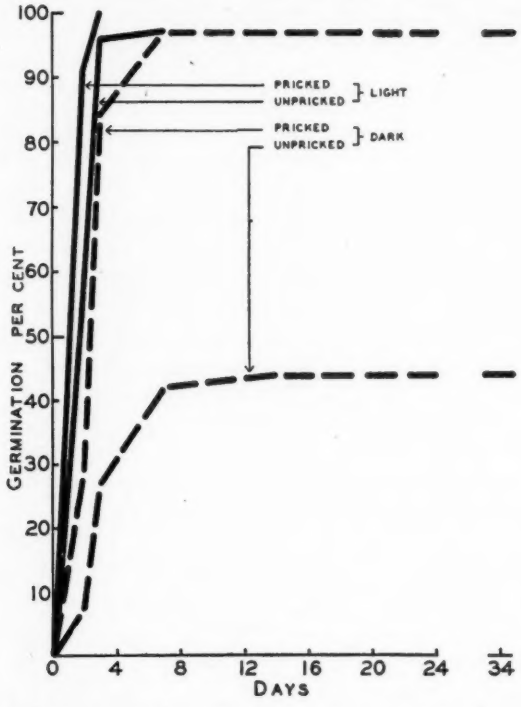

FIG. 8. *Experiment N.S. XIX (continued)—germination in light and darkness of seeds made dormant by alcohol, 3%; pricked and not pricked.*

Expt. N.S. XX

It is a well known fact that high temperature brings about dormancy in lettuce seeds. It was thought of interest to examine whether pricking would offset high temperature dormancy. Accordingly, the following experiment was carried out.

Two lots of 100 seeds, pricked, and 2 × 100 seeds, unpricked, were set between blotters in the dark at a constant temperature of 31° C.

After 46 hr., the unpricked seeds had all become dormant. Of the pricked seeds, two were evidently dead and were removed, while one seed had germinated. The tests were then transferred to low temperature (20° C.) still in the dark where they stayed for the remainder of the test. The results are shown in Fig. 9. The duplicates checked well with each other and have been

averaged. The pricked tests are shown as originating from zero co-ordinates, and have been calculated to percentage of ungerminated seeds at time of transfer to lower temperature.

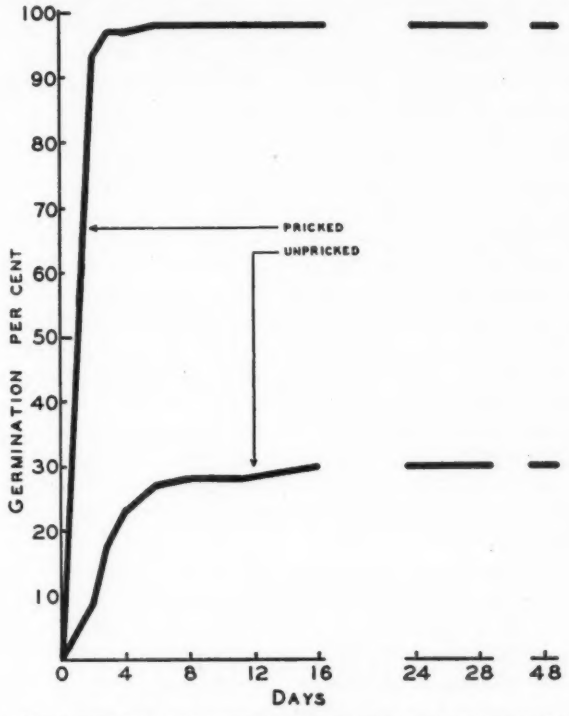

FIG. 9. *Experiment N.S. XX.—germination in darkness of seeds made dormant by high temperature; pricked and not pricked.*

VII. Discussion of Metabolic Possibilities

Since we have felt compelled to reject the suggestion that an anaerobic type of zymasis is brought about by blue light, we may consider another possibility. We have seen that carbon dioxide and alcohol induce dormancy of a type similar to that induced by blue light. It is known that in the presence of carbon dioxide a type of zymasis proceeds that results (in the cases studied) in the production of much acetaldehyde and little alcohol and it is at least possible that a similar type of zymasis would be brought about by means of ethyl alcohol. These suggestions seem to indicate the possibility that the effect of blue light is to bring about a state of a similar type, either through the agency of carbon dioxide or in some other way.

Both on *a priori* considerations and on the grounds that a rising phase of respiratory quotient in the initial stages of germination is a perfectly general

phenomenon with fatty seeds, we may regard the metabolic state of the germinating seed as including a certain amount of zymasis such that under normal conditions some alcohol is produced. If the effect of blue light, as suggested above, is to bring about the same type of zymasis as does carbon dioxide, then we would expect a lower proportion of alcohol in seeds set to germinate in blue light than in those in clear or red light.

Expt. XXI, which was originally planned for another purpose, gives strong support to this hypothesis and is reported below.

Expt. XXIb

Lettuce seed (100 gm.) was spread in a thin layer on wet blotters lining the bottom of a shallow tank (37 in. × 19 in. × 3 in.), which was then covered by a sheet of glass on which was placed a double layer of dark blue cellophane. Above the tank at a height of 4½ ft. was suspended a 500 w. light. The seeds were continuously illuminated for 28 hr., since this experiment was to be checked by XXI*c*, in which seeds were similarly allowed to imbibe but in clear light, and in which it was desirable that the germination process should not be carried too far. The temperature was 22.5° C. above the cellophane. The alcohol content was determined by the Nicloux method. The seeds were distilled in picric acid and two aliquots of the distillate of 5 cc. each were used and titrated with a 19 gm. per liter solution of potassium dichromate after being mixed with an equal volume of concentrated sulphuric acid. Each aliquot required 0.35 cc. of the solution, which corresponded with an alcohol content of 0.008%.

In Expt. XXI*c*, carried out in precisely the same way as XXI*b* except for light color, which in the present case was unscreened, and the fact that the temperature was 24° C., each aliquot required almost exactly 1 cc. potassium dichromate, corresponding to 0.020% alcohol content.

The possibility that the lower alcohol content of the seeds used in XXI*b* is due to the lower temperature of that test must be mentioned. However, the difference is far greater than could be accounted for by an application of the Van't Hoff relation, which gives a corrected value for XXI*b* of 0.009 at 24° C., and we must conclude that a real difference exists. These results support the premise that some zymasis occurs in the normal, germinating seed, as also the suggestion that in blue light the character of the zymasis is changed in such a way that carbon dioxide zymasis predominates.

Acknowledgments

I have to make grateful acknowledgment to Dr. H. B. Sifton under whose guidance this study was carried out, and for whose encouragement and helpful criticism I am much in debt; to Dr. G. H. Duff, for his valuable criticisms and suggestions especially with regard to interpretation of results; to Prof. J. Rogers for help in the alcohol determinations; and also to members of the staff of the Department of Botany for discussions and suggestions that have been of much use.

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